Improvements of B.C.G. immunotherapy in superficial bladder cancer

Peter D.J. Vegt

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IMPROVEMENTS OF BCG-IMMUNOTHERAPY IN SUPERFICIAL BLADDER CANCER

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van de Medische Wetenschappen

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Aan mijn ouders,
mijn schoonouders,
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chapter 1

RATIONALE OF THIS THESIS

RATIONALE OF THESIS

Immunotherapy with Bacillus Calmette-Guérin (BCG) for patients with bladder cancer has been used since 1976.[1] Bacillus Calmette-Guérin is a vaccine containing a live attenuated organism originally developed at the Pasteur Institute of Lille.

In order to use BCG effectively and safely, it is necessary to understand what this vaccine contains, the effect it exerts on the bladder, how to select patients appropriately, how it should be administered and what the results of therapy are likely to be. Most of these aspects of BCG are still unclear, due to the complexity of the immune response to these bacilli which involves stimulation of both cellular and humoral immune reactions.

The majority of patients tolerate BCG-instillations well, but adverse reactions can and do occur. The side effects of BCG-therapy vary from mild malaise and fever till in rare instances life-threatening or fatal sepsis.[2] Because of this toxicity, the indication for intravesical immunotherapy with BCG has to be very strict.

Although BCG-immunotherapy has proved to be very effective in the prophylaxis and treatment of superficial bladder tumors and carcinoma in situ of the bladder,[3] still some patients do not ultimately benefit from this therapy and will show recurrences[4] and progression[5] of their bladder cancer.

To improve the results of intravesical BCG-immunotherapy three aspects are of interest: the toxicity, the efficacy and the indication of the therapy.

The aim of this thesis was to get more insight into the effector mechanism of the BCG nonspecific immunotherapy for patients with superficial bladder cancer, to improve this therapy with regard to toxicity, antitumor efficacy and the indication.

Chapter 2 describes the consensus and controversies of BCG-therapy. From this review it becomes clear that little is known about the effector mechanism of BCG-treatment and that more research is needed in this field. To get more insight into the effector mechanisms of intravesical BCG-therapy, the effects of immunological reactions observed in the urine of patients treated with intravesical BCG is evaluated in chapter 3. Also, the possible effector mechanisms of this therapy in patients with bladder cancer are discussed in this chapter.

BCG is thought one of the most effective agents for the treatment of patients with superficial bladder cancer.[2] The efficacy of intravesical chemotherapy with Mitomycin-C in patients with superficial bladder cancer has been proven.[6,7] To compare the results of intravesical immunotherapy using two different BCG-strains, with intravesical chemotherapy using Mitomycin-C, a prospective randomized trial is described in chapter 4. In this chapter the efficacy of intravesical BCG-Tice and BCG-RIVM administration and intravesical Mitomycin-C administration is evaluated.

Impairment of the toxicity of BCG will result in an improvement of BCG-therapy in patients with bladder cancer. Antituberculostatic drugs, like Isoniazid can prophylactically be administered to decrease the toxicity of BCG-treatment.[8] It is, however, unknown if these drugs will impair the immunologic response of BCG and by that the antitumor effect. In the guinea pig Isoniazid seems to impair the local immunological response.[9] This is the rationale to study the effects of Isoniazid prophylaxis in man. Chapter 5 reports the effects of oral Isoniazid administration on the immunological response of intravesical BCG-therapy in man.

To evaluate the influence of prophylactic Isoniazid administration on the toxicity and efficacy of intravesical BCG-therapy in patients with superficial bladder cancer, the European Organisation for Research and Treatment of Cancer-study 30911 has been designed. In this study the three treatments arms: intravesical BCG and intravesical BCG + prophylactic Isoniazid and intravesical chemotherapy with epirubicin are compared. The interim analysis of the toxicity data available from patients treated with BCG and BCG + Isoniazid in this study is reported in chapter 6 of these thesis.

Improvement of BCG therapy can also be achieved by a more defined indication. With a combination of several conventional prognostic factors, like tumor stage, grade, number of tumors and previous recurrence rates, the risk for tumor recurrence and thus progression can be estimated.[10] However, these factors do not accurately predict the clinical behaviour of the malignant proces.[11] The evaluation of new tumor markers, like p53 and E-cadherin expression may have prognostic value for the individual patient.[12,13] In chapter 7 the prognostic value of p53 and E-cadherin expression are described in patients with superficial bladder cancer.

This thesis concludes with chapter 8 in which the major conclusions are discussed.

REFERENCES

1. Morales A, Eidinger D and Bruce AW (1976): Intracavitary Bacillus Calmette-Guérin in the treatment of superficial bladder tumors. J Urol 116: 180-183.

2. Lamm DL, Meijden APM van der, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A and Debruyne FMJ (1992): Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial bladder cancer. J Urol 147: 596-600.

3. Brosman, SA (1992): Bacillus Calmette-Guérin immuno-therapy, techniques and results. Urol.Clin.N.Am. vol. 19: 557-564.

4. Witjes JA, Meijden APM van der, Witjes WPJ, Doesburg W, Schaafsma HE, Debruyne FMJ (1993): A randomized prospective study comparing intravesical instillations of Mitomycin-C, BCG-Tice and BCG-RIVM in pTa-pT1 tumors and primary carcinoma in situ of the urinary bladder. Eur.J.Cancr. vol. 29A. no. 12.1672.

5. Herr HW (1991): progression of stage T1 bladder tumors after intravesical bacillus Calmette-Guérin. J Urol 145: 40-44.

6. Huland H, Otto U, Droese M and Kloeppel G (1984): Long-term mitomycin C instillation after resection of superficial bladder carcinoma: influence on recurrence, progression, and survival. J Urol 132: 27.

7. Bracken B, Johnson D, Von Eschenbach A, Swanson D, De Furia D and Crook S (1980): Role of intravesical mitomycin in management of superficial bladder tumors. Urology 16: 10.

8. Lamm DL, Steg A, Boccon-Gibbon L, Morales A, Hanna MG, Pagano F, Althan O, Bosman S, Fisher HAF, Jakse G, Chisholm GD, Meijden APM van der, Debruyne FMJ (1989): Complications of bacillus Calmette-Guérin immunotherapy: review of 2602 patients and comparison of chemotherapy complications. In EORTC Genito-urinary Group Monograph 6: BCG in superficial bladder cancer: 335-355. Alan R. Liss, Inc..

9 De Boer LC, Steerenberg PA, Meijden APM van der, Van Klingerden B, De Jong WH, Elgersma A, Debruyne FMJ, Ruitenberg EJ (1992): Impaired

immune response by Isoniazid treatment during intravesical BCG-administration in the guinea pig. J Urol 148: 1577-1582.

10. Witjes JA, Kiemeney LALM, Oosterhof GON, Debruyne FMJ (1992): Prognostic factors in superficial bladder cancer. A review. Eur. Urol 21:89.

11. Kiemeney LALM, Witjes JA, Heijbroek RP, Verbeek ALM, Debruyne FMJ (1993): Predictability of recurrent and progressive disease in individual patients with primairy superficial bladder cancer. J Urol 150:63.

12. Sarkis AS, Dalbagni G, Cordon-Cardo C, Zhang ZF, Sheinfeld J, Fair WR, Herr HW, Reuter VE (1993): Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. J.Nath.Cancer Inst. 85:53.

13. Briguier PP, Umbas R, Schaafsma HE, Karthaus HFM, Debruyne FMJ, Schalken JA (1993): Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. Cancer Res. 53:3241.

BACILLUS CALMETTE-GUÉRIN IN SUPERFICIAL BLADDER CANCER: CONSENSUS AND CONTROVERSIES

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ABSTRACT

In this overview, Bacillus Calmette-Guérin (BCG) immunotherapy in superficial bladder cancer items are discussed on which consensus has been reached and on which controversies exist. The evaluation of the optimal route of administration has shown that intravesical instillation of BCG alone is accepted as the best route of administration. In searching for the appropriate BCG-strain, the analysis of the results of 7 substrains has made clear that no particular strain has shown superiority over others. In finding the optimal treatment schedule there is strong evidence that maintenance therapy is superior to induction therapy alone. No consensus has been reached about the optimal dose for BCG-therapy nor about how the toxicity of BCG-treatment can be reduced. Although some reports have stated that BCG-immunotherapy is superior to chemotherapy for the treatment of patients with superficial bladder cancer, more data are needed to prove this statement.

In conclusion: although BCG has been proven to be very effective in the treatment of patients with superficial bladder cancer, it is certainly not a panacea for all patients with superficial bladder cancer.

INTRODUCTION

Bacillus Calmette-Guérin (BCG) has been used for over 15 years now as an adjuvant treatment for superficial bladder cancer. At the start of this new therapy in 1976 by Morales, a number of controversies existed. It was unknown which dose was optimal, which treatment scheme should be used and whether there was an optimal BCG-strain. No consensus existed about the appropriate route of administration, about which tumors should be treated and which not. There were problems about the toxicity of BCG intravesical therapy and how to deal with it. The working mechanism of BCG was not understood. At present, non specific immunotherapy with BCG for superficial bladder cancer is considered one of the most efficacious treatments. It is probably the most widely used and most successful immunotherapy in man. This article describes the items on which we have reached consensus as well as the problems which still are unsolved or on which controversies still exist. It describes the evolution of BCG-therapy over the last 15 years.

Calmette and Guérin developed in 1908 a vaccine for the prevention of tuberculosis. This widespread contagious disease had caused millions of deaths at that time. These 2 French co-workers were working with a highly virulent strain of the bovine tubercle bacillus. By transplanting it from culture to culture the tubercle bacillus was gradually losing its noxious characteristics.[1] Calmette and Guérin spent 13 consecutive years and 231 transplantations to tame the bovine bacillus. It had become completely harmless but its antigenic properties were unimpaired. Since 1921, over 500 million vaccinations with BCG to prevent tuberculosis have been performed.

In the 1930s, Rosenthal[2] reported that when the vaccine was administered to animals by various routes, a local and generalized stimulation of the immune apparatus could be observed. This stimulation led to the eradication of several tumors in animals.

The research group of Zbar and Rapp[3] investigated the basic principles for successful nonspecific active immunotherapy in a guinea pig hepatocarcinoma. By intratumoral injection of BCG, not only the primary tumor but also metastases in the regional lymph nodes were eradicated. Furthermore, the existence of tumor immunity could be demonstrated as BCG-cured animals rejected a second tumor cell challenge.

In man, immunotherapy with BCG was first used by Mathé et al.[4] in the treatment of acute lymphoblastic leukaemia.

Morales et al.[5] were the first to investigate the efficacy of BCG administered both as an intradermal application and intravesically diluted in saline in patients with superficial transitional urinary bladder tumors. This therapy turned out to be very successful and at present urologists all over the world are using it. However, BCG-therapy has its indications and also its limits. BCG has its place together with intravesical chemotherapy but does not replace it completely. BCG is certainly not a panacea for every patient with superficial bladder cancer. Since this therapy seems to have more side effects than intravesical chemotherapy, a careful risk-to-benefit analysis has to be made about which patient should receive BCG and which not.

THE ROUTE OF ADMINISTRATION

Five different routes of BCG-administration for the treatment of superficial bladder cancer have been used so far:
- percutaneous administration
- intralesional injection
- oral administration
- intravesical instillation combined with percutaneous administration
- intravesical instillation alone

Percutaneous administration of BCG in superficial bladder cancer was investigated by Martinez-Pineiro and Muntanola.[6 7] Per week, 10 scarifications measuring 5 cm in length were administered. At each treatment 0.5 ml of lyophilized Pasteur BCG vaccine, containing 300 mg/ml was used. Only 7 patients were treated and many variable factors (different periods of treatment, patients with or without residual bladder tumors) made it impossible to estimate the value of this intensive treatment but an antitumor effect was observed. Intralesional administration was performed by the same authors in two patients by injecting 37.5 mg of BCG into their bladder tumor. One patient developed a serious hypersensitivity reaction, resulting in an allergic shock. Thereafter, this way of administration was abolished.

The antitumor effect of oral administration of BCG was studied by several investigators (Lamm personal communications 1987 and 1988). Netto and Lemos[8] treated 10 patients with muscle-invasive bladder cancer using fresh BCG of the Moreau strain in high doses. A complete response was obtained in 7 patients. Lamm (personal communications 1978, 1988) used oral administration of the Tice-BCG-strain in muscle invasive bladder cancer in patients who could not be treated by radiation of cystectomy. Although tumor

regression was observed, the results were less impressive compared with those of Netto and Lemos.[8] Lamm et al.[9] also investigated the effect of orally administered BCG (200 mg Tice-BCG, 3 times per week) in superficial bladder tumors. No measurable antitumor effect, however, was observed in patients treated this way.

The most thoroughly investigated route of administration is the intravesical instillation, initially combined with percutaneous administration. Morales et al.[5] developed an empirically based therapeutic scheme which remained the treatment modality in superficial bladder cancer for many years. They instilled BCG in a watery solution in the bladder once weekly for 6 consecutive weeks. At the same time, BCG was administered percutaneously at the anterior surface on the thigh by a multiple puncture apparatus After 10 years of experience, reviewing his results, Morales and Nickel[10] reported a complete response in 50% of the patients using BCG as a prophylactic treatment after transurethral resection. In 58% of patients with residual disease and in 77% of patients with carcinoma in situ, complete response was achieved. Two prospective randomized clinical trials,[11, 12] using the Morales treatment schedule, were conducted in the USA. Responses were in the same range as those obtained by Morales. Herr et al.[13,14] used intravesical and percutaneous BCG for the treatment of carcinoma in situ. A complete response was noted in 65% of the patients.

Excellent results of intravesical BCG-instillation without percutaneous administration were reported by Brosman.[15, 16] In a randomized study, patients were treated either with Thiotepa instillations or with BCG-instillations; BCG was administered intravesically weekly for 12 consecutive weeks, followed by instillations every 2 weeks for 3 months and thereafter every month until a recurrent tumor developed or the patient had been treated for a total period of 2 years. No recurrences have been observed in BCG-treated group, while 40% in the Thiotepa group suffered from recurrences.

Treating carcinoma in situ, 94% of the patients were rendered free of tumor.[17] Brosman[15 16] stated that patients do not require presensitization or continued intradermal inoculations of BCG as he observed that all his patients, when treated with intravesical instillations alone, converted from a negative PPD skin reaction to a positive one after 9 instillations. A problem in these studies was the increasing toxicity of the prolonged instillation treatment.

At the moment, percutaneous supportive inoculation has been abandoned. It is considered as not essential for the antitumor effect of BCG-immunotherapy in superficial bladder cancer, but the value of percutaneous administra-

tion has not been intensely investigated. Thus, at the moment, intravesical administration of BCG alone is accepted in most centers to be the best route of administration in treating patients with superficial transitional cell carcinoma of the urinary bladder.

APPROPRIATE STRAIN

Originally all of the known BCG-strains are derived from the Pasteur strain developed by Calmette and Guérin. From the 1930s on, cultures of the Pasteur strain were imported in several countries all over the world. Mostly they were exported to the various National Institutes for the development of antituberculous vaccines. By culturing from year to year substrains were obtained which showed resemblance but also differences with the original Pasteur strain in genetical and immunological properties. Comparative studies of different strains have been performed for several animal tumor models but for superficial bladder cancer very few studies have been carried out. The efficacy of BCG depends on at least two factors: the number of bacilli and the viability. The viability is the ability of the bacilli to multiply in vivo. The effect of BCG viability on treatment results was investigated for superficial bladder cancer by Kelley et al. in 1985.[17] In the early 1980s, marked differences were noted between different strains and even between lots of BCG of the same strain. The number of culturable particles ranged from 5×10^6 to 1×10^{12} per ampule.

At present, all manufacturers are able to deliver a product with a relative constant quality, viability, and number of living microorganisms. However, since BCG is a living product, exact measures and limitations are requested to store and to use BCG.

So far, the results of 7 substrains used for immunotherapy in superficial bladder cancer have been published. These preparations are Pasteur (France), Armand-Frappier (Canada), Tice (USA), Connaught (Canada), Evans (UK), Moreau (Brasil) and RIVM (The Netherlands). In the meantime, countries in Eastern Europe and in Asia have begun to use their own BCG substrains as well.

In culturing BCG bacilli, the mycobacteria usually are grown as a pellicle on the surface of a liquid medium. At harvesting, the pellicle is ground in a ball mill to a paste. The final product contains not only living BCG bacilli but also dead microorganisms and subcellular debris. It is still unknown whether the debris and crushed bacilli play a role in the efficacy of BCG against superficial bladder tumors.

There is another method of growing the bacteria in a so-called homogeneously stirred deep culture system. This culture method results in a relatively high ratio of viable organisms and a small quantity of subcellular debris and dead bacilli. After both methods of culture, the vaccines are harvested and lyophilized. Most of the BCG preparations are surface cultures. Evans strain and the Dutch RIVM-strain are produced according to the homogeneous culture method.

In 1988, a prospective randomized study comparing the efficacy of BCG-RIVM versus Mitomycin-C was reported by Debruyne et al..[18] At that time it was the first study reported in which BCG had been compared with Mitomycin-C. There was no difference in efficacy between the two drugs but it must be stated that the treatment schedule was different for the two arms. BCG patients were treated with a 6-week schedule while Mitomycin-C patients received a 6 months schedule. The results had led to the assumption that the Dutch BCG-RIVM might be inferior to other strains for two reasons. Firstly, the efficacy of BCG-RIVM was not higher than of a chemotherapeutic agent (Mitomycin-C) and secondly, the side effects reported seemed less significant compared to other strains.

To investigate the quality of BCG-RIVM, the Dutch Southeast Cooperative Urological Group decided to embark upon a three-arm prospective randomized trial in which BCG-RIVM, BCG-Tice and Mitomycin-C were com- pared. This was the first prospective randomized study in the world in which two BCG-strains were compared with Mitomycin-C in patients with primary or recurrent superficial bladder tumors, including carcinoma in situ. Therapeutic regimens were as follows: after complete transurethral resection of all visible tumors in the Mitomycin-C group, 30 mg Mitomycin-C in 50 ml saline was instilled once a week for 4 weeks and thereafter once a month for a total of 6 months. In the BCG groups 5×10^8 colony forming units in 50 ml saline of Tice respectively RIVM strain were instilled once a week for 6 consecutive weeks. The median follow up is 36 months (range 2-81). The analysis of efficacy for the papillary tumors shows that Mitomycin-C and BCG-RIVM treatments were equally effective ($p=0.53$), BCG-Tice was less effective than BCG-RIVM and Mitomycin-C ($p = 0.07$ respectively $p = 0.01$). The side effects of the BCG-strains were comparable, both locally and systemically [33]

As there is only one prospective study with sufficient patient numbers reported, more studies are needed to investigate whether there is an optimal BCG-strain.

THE OPTIMAL TREATMENT SCHEDULE

Basically two different instillation schedules have been used so far. Morales started with a so-called induction scheme of 6 consecutive weekly instillations. The second scheme does comprise the 6-week induction course but is followed by repeated instillations during months up to 3 years although in the meantime the patient is free of tumor. The theory behind these repeated instillations is the assumption that these "booster" instillations evoke a renewed immune response against the possibly developing bladder tumors in the patient. This instillation scheme that has been used most frequently in large prospective trials in the USA is the so called "maintenance"-scheme.[19] In this scheme, 6 weekly instillations are administered followed by 3 consecutive weekly instillations at 3 months, at 6 months and thereafter every 6 months up to 36 months. This means that a patient who stays tumor free after the initial resection will receive a total of 27 instillations in a period of 3 years. Few studies have been performed to investigate the efficacy of a 6-week schedule versus maintenance treatment. Hudson et al.[20] reported no difference in efficacy between a 6-week schedule and maintenance therapy but prolongation of toxicity was observed with maintenance BCG-therapy. The series of 42 patients investigated, however, was small.

The effect of repeated 6-week courses, after failure of the first induction course, was also investigated by Catalona et al..[21] Of 100 consecutive patients with superficial bladder cancer treated with 6 weeks of BCG, 44 became free of tumor. Of 49 patients treated with a second course of BCG, 19 were consequently rendered free of tumor. However, among patients who had failed 2 or more courses of BCG-therapy the risk of invasive (30%) or metastatic (50%) cancer exceeded the prospects for eradicating the superficial tumors (20%) with further therapy.

The largest study comparing 6 weeks of BCG with a maintenance schedule of BCG has been carried out by the South West Oncology Group.[22,23] After an interim analysis of 391 patients randomized between non-maintenance versus maintenance, a significant superiority was found in favor for maintenance therapy. This was observed for 270 patients with Ta and T1 tumors ($p=0.0001$) but also for 121 patients with carcinoma in situ ($p=0.04$).

Thus, there is strong evidence that maintenance therapy following successful induction therapy provides better protection against recurrences than induction therapy alone.

Regarding the results of the reported literature, it can be concluded that of the different instillation schemes used so far, maintenance BCG-immunotherapy is superior.

ADVERSE EFFECTS OF BCG

Compared to intravesical chemotherapy, instillations with BCG provoke more local and systemic side effects. In addition to the commonly induced granulomatous inflammatory reactions in the bladder, which produce irritative symptoms, BCG-therapy may cause systemic side effects varying from mild malaise and fever to, in rare instances, life threatening or fatal sepsis. Without doubt these possible side effects have led to the decision of many urologists not to use BCG in every patient who needs intravesical instillation therapy. This means that not in every country BCG has been accepted as the first-line therapy for superficial tumors.

In treating the adverse effects of BCG-treatment, options vary according to the severity of the toxicity from delaying or withholding instillations to treatment with antituberculous drugs for up to 6 months (Table 1). However, in general, 95% of the patients have no serious side effects. The recognition of risk factors, particularly traumatic catheterization or concurrent cystitis, that may result in systemic BCG absorption as well as the prompt and appropriate treatment of early side effects should significantly decrease the incidence of severe toxicity.[24]

It is difficult to distinguish between therapy-related symptoms that may indicate efficacy of BCG and symptoms that may represent adverse effects. Many investigators believe that an inflammatory reaction is an important component of the response to BCG-therapy. In case of bladder-irritative symptoms, some investigators stop instillations because they consider this phenomenon a side effect while others continue the instillations presuming that by evoking inflammation, BCG is working optimally in that particular patient.[25] Because the overall side effects of BCG-instillations are more pronounced than of chemotherapeutic drugs, attempts have been made to reduce the toxicity of BCG-therapy without affecting the efficacy of it.

BCG is generally highly sensitive to antituberculous drugs of which Isoniazid, Rifampin, streptomycin and Ethambutol are well known. Of all antituberculous drugs Isoniazid is used most frequently. As these tuberculostatic agents are capable of curing patients with severe BCG-induced toxicity including

BCG-sepsis, it might be that these agents, if used prophylactically can reduce side effects significantly. In an animal study, the administration of oral Isoniazid has shown to impair the immune response after intravesical BCG-instillation.[26] In humans, Isoniazid administration did not impair the local immune response after BCG-treatment.[27] Because no data are available concerning the impact of Isoniazid on the antitumor efficacy of intravesical BCG in patients with superficial bladder tumors, the prophylactic use of antituberculous drugs is subject of investigation in several trials.

A second method to reduce toxicity can be achieved by reducing the dose of BCG. However, also here the dilemma will be the same as for antituberculous drugs: Will the efficacy be maintained if toxicity decreases?[28]

Table 1. Treatment recommendations for BCG-related complications.

Fever less than 38.5°C	No instillation Hold BCG until symptoms have resolved
Fever greater than 38.5°C for 12-24 hours	No instillation Isoniazid at 300 mg daily for 3 months May resume BCG when asymptomatic
Allergic reactions	Isoniazid at 300 mg daily for 3 months Further BCG only if benefit exceeds risk
Acute severe illness	Isoniazid at 300 mg. Rifampin at 600 mg Ethambutol at 1200 mg, daily for 6 months No further BCG
BCG sepsis	Isoniazid at 300 mg, Rifampin at 600 mg Ethambutol at 1200 mg daily for 6 months Consider Prednisolone at 40 mg intravenously and acutely

THE OPTIMAL DOSE

Several studies are now ongoing to investigate the efficacy and toxicity of low dose BCG. A low dose (75 mg) of the Pasteur strain was compared to the standard dose (150 mg) by Pagano et al..[28] The preliminary results indicate similar response rates in 87 patients. The recurrence-free rates were 90.5% (low dose) and 86.4% (standard dose) respectively. The incidence of local and systemic side effects, however, are significantly less in the low dose group. Cystitis was observed in 31% (low dose) and 54% (standard dose), while fever was noted in 14% and 26% respectively. Two dose-related studies have

been published in the USA. Morales et al.[29] reported that a half dose (60 mg) of Armand Frappier BCG resulted in a reduction in overall efficacy: from 67% success in 45 patients treated with 120 mg BCG to 39% in 41 patients treated with 60 mg BCG ($p < 0.02$). As BCG is a living preparation it is expected that storage of BCG will inevitable lead to reduction of the number of viable microorganisms. In a retrospective review performed by Blumenstein et al.,[30] no evidence was found of reduced efficacy with reduced dose being estimated by the decline of colony forming units which occurs with shelf storage. The dose of most preparations is given by most manufacturers in milligrams and in colony forming units. It is advised to use per instillation: 150 mg of Pasteur, Armand Frappier 120 mg, Connaught 120 mg and Tice 50 mg.

The number of colony forming units in one ampule varies between manufacturers and even between lots of the same substrain. The optimal dose at present ranges between about 3×10^8 and 1×10^9 colony forming units. The living bacteria are present as solitary bacilli but also as clumps containing 5, 50 or even 500 bacilli. These clumps all grow in culture as if they developed from one solitary bacillus, ultimately forming one colony in the culture disk. In practice the number of colony forming units does not inform about the real number of bacilli present. It is also unknown whether a clump of bacilli acts differently compared to a solitary bacillus, with regard to immune stimulation when it adheres to the bladder wall.

Regarding this information it is questionable whether a dose reduction of 50% could have any impact at all. Probably a fourfold or more dose reduction should be investigated to address the item of dose reduction.

IS INTRAVESICAL IMMUNOTHERAPY SUPERIOR TO INTRAVESICAL CHEMOTHERAPY?

The question whether intravesical immunotherapy with BCG is superior to intravesical chemotherapy remains actual. There is no doubt about the efficacy of BCG in eradicating carcinoma in situ or preventing papillary tumors after transurethal resection. Although series are not large and follow-up is too short, several reports indicate that BCG is capable to prevent muscle invasive disease.[31,32] More data are needed to prove this observation. If the progression rate is lower, as assumed in low risk or intermediate risk tumors, the other important objective is to prevent recurrences. Recurrences are delayed using intravesical chemotherapeutics as well as BCG. However, BCG is more toxic and therefore the urologist has to make a risk to benefit analysis

for the individual patient. Should he select a potent drug with pronounced adverse effects or a less potent drug with virtually no side effects?

CONCLUSION

Intravesical therapy with BCG has proven to be very effective in the prophylaxis and treatment of superficial bladder tumors and carcinoma in situ. In most series, BCG is superior to intravesical chemotherapy in bladder cancer therapy. Because of the more pronounced side effects, the risk and benefit for the individual patient should be considered in individual patients. This means that for low- and intermediate-risk patients there seems to be a role for intravesical chemotherapy.

BCG should not be administered to patients with low stage, low grade (stage pTa, grade I) unifocal or primary tumors. In patients with intermediate risk (50% of all patients) it is questionable, whether one should choose between effective chemotherapeutic drugs or BCG. In high risk patients (15%) BCG is therapy of first choice. In this category of patients the benefit of the therapy exceeds the risk of possible side effects and the disadvantage of long-term treatment. Certainly this is true for carcinoma in situ.

A number of issues around BCG-therapy seems to have reached consensus now. An optimal route of administration has been defined as well as an optimal instillation scheme. Too few studies have been performed to reveal the best strain if there is any. Studies about the optimal dose are ongoing as well as studies to address the toxicity. It must be remembered, however, that BCG never will be the best treatment option for every patient with superficial bladder cancer.

REFERENCES

1. Guérin C (1980): The history of BCG. In: Rosenthal SR (ed): BCG vaccine: tuberculosis-cancer. Littleton Massachusetts: P.S.G. Publishing Company Inc. p. 37.

2. Rosenthal SR (1938): The general tissue and humeral response to an avirulent tubercle bacillus. In: Illinois Medical and Dental Monographs. University of Illinois, Press, Urbana, Illinois.

3. Zbar B, Bernstein ID, Bartlett GL, Hanna MG Jr and Rapp HJ (1972): Immunotherapy of cancer: regression of intradermal tumors and prevention of growth of lymph node metastases after intralesional injection of living mycobacterium bovis. J Natl Cancer Inst 49: 119-130.

4. Mathé G, Amiel J, Schwartzenberg L, Schneider M, Cattan A, Schlumberger J, Hayat M and De Vassal F (1969): Active immunotherapy for acute lymphoblastic leukaemia. Lancet i: 697-699.

5. Morales A, Eidinger D and Bruce AW (1976): Intracavitary Bacillus Calmette-Guérin in the treatment of superficial bladder tumors. J Urol 116: 180-183.

6. Martinez-Pineiro JA and Muntanola P (1977): Non-specific immunotherapy with BCG vaccine in bladder tumors. A preliminary report. Eur Urol 3: 11-22.

7. Martinez-Pineiro JA (1984): BCG vaccine in superficial bladder tumors: eight years later. Eur Urol 10: 93-100.

8. Netto NR Jr and Lemos CG (1984): Bacillus Calmette-Guérin immunotherapy of infiltrating bladder cancer. J Urol 132: 675-677.

9. Lamm DL, Pilot S and Sardosi MF (1986): Oral Bacillus Calmette-Guérin versus intravesical plus percutaneous Bacillus Calmette-Guérin in superficial transitional cell carcinoma. J Urol 135: 186A.

10. Morales A and Nickel JG (1986): Immunotherapy of superficial bladder cancer with BCG. World J Urol 3: 209-214.

11. Camacho FJ, Pinsky CM, Kerr D, Whitmore WF and Oettgen HF (1980): Treatment of superficial bladder cancer with intravescial BCG. Proc Amer Ass Cancer Res and Amer Soc Clin Oncol 21: 359.

12. Lamm DL, Thor DE, Winters WD, Stogdill V and Radwin HM: BCG-immunotherapy of bladder cancer (1981): inhibition of tumor recurrence and associated immune responses. Cancer 48: 82.

13. Herr HW, Pinsky CM, Whitmore WF Jr, Oettgen HF and Melamed MR (1983): Long-term effect of intravesical BCG on carcinoma in situ of the bladder. Cancer 51: 1323-1326.

14. Herr HW, Pinsky CM, Whitmore WF Jr, Soagani PC, Oettgen HF and Melamed MR (1986): Long-term effect of intravesical Bacillus Calmette-Guérin on flat carcinoma in situ of the bladder. J Urol 135: 265-267.

15. Brosman SA (1982): Experience with Bacillus Calmette-Guérin in patients with superficial bladder carcinoma. J Urol 128: 27-30.

16. Brosman SA (1985): The use of Bacillus Calmette-Guérin in the therapy of bladder carcinoma in situ. J Urol 134: 36-39.

17. Kelley DR, Ratliff TL, Catalona WJ, Shapiro A, Lage JM, Bauer WC, Haaff EO and Dresner SM (1985): Intravesical Bacillus Calmette-Guérin therapy for superficial bladder cancer: effect of Bacillus Calmette-Guérin viability on treatment results. J Urol 134: 48-53.\

18. Debruyne FMJ, Meijden APM van der and Geboers ADH (1988): BCG-RIVM versus Mitomycin-C intravesical therapy in patients with superficial bladder cancer. Urology 31: (suppl), 20-25.

19. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P (1991): A randomized trail of intravesical doxorubicin and immunotherapy with bacille Calmette-Guérin for transitional cell carcinoma of the bladder. N Engl J Med 325: 1205-1209.

20. Hudson MA, Ratliff TL, Gillen DP, Haaff EO, Dresner SM and Catalona WJ (1987): Single course versus maintenance bacillus Calmette Guérin therapy for superficial bladder tumors: A prospective randomized trial. J Urol 138: 295-298.

21. Catalona WJ, Huson M'Liss A, Gillen DP, Andriole GL and Ratliff TL (1987): Risk and benefits of repeated course of intravesical Bacillus Calmette-Guérin therapy for superficial bladder cancer. J Urol 137: 220-224.

22. Martinez Pineiro JA, Jimenez Léon J and Martinez-Pineiro L J et al. (1989): Intravesical therapy comparing BCG Adriamycin and Thiotepa in 200 patients with superficial bladder cancer: a randomized prospective study. In: Debruyne FMJ, Denis L and Meijden APM van der (eds), EORTC Genito-Urinary Group Monograph 6: BCG in superficial Bladder Cancer. New York, Liss: 237-252.

23. Lamm DL (1992): Maintenance versus no-maintenance BCG-immunotherapy of superficial bladder cancer: J Urol 147: 4, 274a.

24. Lamm DL, Meijden APM van der, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A and Debruyne FMJ (1992): Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial bladder cancer. J Urol 147: 596-600.

25. Brosman SA, Lamm DL, Meijden APM van der and Debruyne FMJ (1989): A practical guide to the use of intravesical BCG for the management of Stage Ta, T1, CIS transitional cell cancer. In: Debruyne FMJ, Denis L and Meijden APM van der (eds), EORTC Genito-Urinary Group Monograph 6: BCG in Superficial Bladder Cancer, New York, Liss: 311-323.

26. De Boer LC, Steerenberg PA, Meijden APM van der, Van Klingerenden B, De Jong WH, Elgersma A, Debruyne FMJ, Ruitenberg EJ (1992): Impaired immune response by Isoniazid treatment during intravesical BCG-administration in the guinea pig. J Urol 148: 1577-1782.

27. Stassar MJJG, Vegt PDJ, Steerenberg PA, Van der MeijdenAPM, Meiring HD, Dessens-Kroon M, Geertzen HGM, Den Otter W (1994): Effects of Isoniazid (INH) on the BCG-induced immunne response after intravesical BCG-therapy for superficial bladder cancer. Urol.Res. 22: 177-184.

28. Pagano F, Bassi P, Milani C, Meneghini A, Maruzzi D and Garbeglio A (1991): A low dose bacillus Calmette-Guérin regimen in superficial bladder cancer therapy: Is it effective? J Urol 146: 32-25.

29. Morales A, Nickel JC and Wilson JWL (1992): dose-response of bacillus Calmette-Guérin in the treatment of superficial bladder cancer. J Urol 147: 1256-1258.

30. Blumenstein BA, Lamm DL, Jewett MA, Humphries G, Boux L, Montie JE, Scardino P (1990): Effect of colon-forming unit dose of Connaught BCG outcome to immunotherapy in superficial bladder cancer: A SWOG study. J Urol 143: 340A.

31. Herr, HW (1988): Bacillus Calmette-Guérin therapy alters the progression of superficial bladder cancer. J.Clin.Oncol. vol.6, 1450.

32. Sarosdy MF (1992): principles of intravesical chemotherapy and immunotherapy. Urol.Clin N.America. vol.19: 509-519.

IMMUNOLOGICAL PRODUCTS IN THE URINE OF SUPERFICIAL BLADDER CANCER PATIENTS AFTER INTRAVESICAL IMMUNOTHERAPY WITH BACILLUS CALMETTE-GUÉRIN

Adapted from:
Leucocytes in the urine after intravesical BCG-treatment for superficial bladder cancer: a flow cytofluorometric analysis

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Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guérin

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Cancer Immunol. Immunother. (1991) 33: 411-416.

Induction of urinary IL1, IL2,IL6 and TNF during intravesical immunotherapy with BCG in superficial bladder cancer

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Cancer Immunol. Immunother. (1992) 34: 306-312.

INTRODUCTION

Bacillus Calmette-Guérin (BCG) is an attenuated strain of Mycobacterium bovis commonly used for vaccination against tuberculosis. The use of BCG for immunotherapy of cancer has been extensively investigated, especially in the 1970s.[1]

Laboratory studies have shown that BCG-administration can result in tumor regression and even tumor-specific immunity in several animal tumor systems. However, clinical use of BCG-immunotherapy in humans has been disappointed, except in superficial bladder cancer.[2] Several independent clinical trials have proven that intravesical BCG-treatment is efficacious.[3] BCG reduces the recurrence rate after endoscopic surgery of papillary tumors. Moreover, BCG has become the drug of choice for the treatment of carcinoma in situ of the bladder urothelium and cures 60-70% of the patients.[3] Importantly, intravesical BCG-treatment possibly may reduce progression of tumor grade and stage.[4]

From the tumor-immunological point of view, immunotherapy seems indicated for transitional cell carcinoma of the bladder, since evidence exists that bladder cancer may be immunogenetic.[5,6]

Depending on the route of administration, BCG is known to stimulate various parts of the immune system both at the humoral and at the cellular level. The cell-mediated immunity seems to be important for the activity of BCG as an immunotherapeutic agent against cancer.[2,7] For treatment of superficial bladder carcinoma, BCG is repeatedly administered in the bladder cavity.

This local application usually results in systemic immunity to BCG, which can be measured by a delayed type hypersentivity (DTH) reaction to purified protein derivate (PPD) in the skin.[8] In the bladder wall of patients, mononuclear cell infiltrates with granunolomatous characteristics are induced after repeated BCG-administration. Recent immunohistochemical studies of these BCG-induced reactions have shown that the major infiltrating cell type is the T-lymphocyte. Macrophages and B-lymphocytes have been found in lower amounts. Induction of Major Histocompatibility Complex Class II (HLA-DR) antigen expression on urothelial cells has also been reported.[9]

The mechanisms by which BCG exerts its antitumor activity are not clearly understood.[7] It may be that the induction of the PPD skin reaction and granulomatous bladder wall infiltrates are associated with the clinical response of patients.[8,10] In animal models, T-lymphocytes also seem to play a crucial role in the mode of action of BCG.[11,12]

To gain inside in the antitumor activity of BCG, the histopathological reactions induced by intravesical BCG-administration in the guinea pig model have been studied.[13]

To evaluate immunological reactions in patients with superficial bladder cancer treated with BCG the immunological products in the urine of these patients were analysed. This is a non-invasive alternative for the study of immunological reactions in comparison with histopathological studies. The desquamated leucocytes from the bladder wall present in spontaneously voided urine of patients after BCG-treatment have been studied. Also the presence of the cytokines: Interleukine1 (IL1), Interleukine2 (IL2), Interleukine6 (IL6) and Tumor Necrosis Factor α (TNFα), which are products of leucocytes present in the bladder wall, was investigated in the urine of these patients.

MATERIALS AND METHODS

Patients with superficial bladder cancer were treated with weekly instillations for 6 consecutive weeks. BCG (RIVM-strain) was used in a dose of $5x10^8$ colony forming units per instillation.

Leukocytes from urine

Voided urine was collected before (T_0) and approximately 24 h (T_{24}) and 48 h (T_{48}) after BCG-instillation number 1 (2 patients) and after 5 and more instillations (8 patients). The mean percentages of viable cells was determined by trypan blue (0.5%) exclusion.

Immunofluorescence staining

For fluorescence-activated cell sorter (FACS) analysis, cells ($5x10^5$) were labelled with the following monoclonal antibodies: FK32 (anti-CD15, granulocytes), anti-HLe1 (anti-CD45, leucocytes); anti-Leu-M3 (anti-CD14, monocytes/macrophages); anti-Leu-12 (anti-CD19, B-lymphocytes); anti-Leu-4 (anti-CD3, T-lymphocytes); anti-Leu-2(anti-CD4,helper/inducer T-lymphocytes); anti-Leu-3 (anti-CD, helper/inducer-lymphocytes); anti-Leu-2 (anti-CD8, suppressor/ cytotoxic T-lymphocytes); anti-Leu-11c (anti-CD16, NK-cells); anti-Leu-19 (anti-CD56, NK-cells); anti-IL2-R (anti-CD25, IL-2receptor); anti-HLA-DR (Ia nonpolymorphic).

Flow cytofluorometry and analysis

Twelve thousand cells were measured with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA,USA) and analysis of the data was performed with FACScan software on a Packard 9920S computer.

Lymphocyte subpopulations were determined with a selective cell measurement procedure.[15]

Detection of IL1, IL2, IL6 and TNFα

Determination of IL1, IL2, IL6, TNFα and IFNγ in urine specimens was performed in urines collected before instillation and 2, 4, 6, 8 and 24 hours thereafter, during 6 weekly instillations. Urine was immediately frozen to -20°C. Afterwards it was thawed, centrifuged, dialysed and sterilized. Thereafter samples were stored (-20°C) until determination of cytokines.

IL1 was measured with the T-cell line D10(N4)M. The nature of the IL1 in some urine samples was investigated by neutralization tests. For detection of IL2 a specific bioassay with the IL2-dependent murine T-cell line CTLL-16 was used. IL6 was measured with the hybridoma growth factor assay B9. TNFα was determined with a TNFα specific ELISA. IFNγ was measured with a commercially available ELISA (Holland Biotechnology, Leiden, The Netherlands). The detection limits of the various assays were 6 U IL1, 0.5 U IL2, 2 U IL6, 50 pg TNFα and 1 U IFNγ per ml urine. The results were standardized to urine creatinine (U/μmol or pg/mmol creatinine) so that sample data were comparable, regardless of the urine volume. The mean creatinine concentration for all samples was 7.6 ± 3.9 μmol/ml urine (n=195).

RESULTS

Leucocytes in the urine

The presence of leucocytes in the urine was studied in 7 patients with superficial bladder cancer who were treated with BCG.

The total number of cells in urine was markedly increased at 24h and 48h after five or more BCG-instillations (Figure 1), indicating a local cellular reaction of the immune system induced by BCG. At all times investigated, the majority of the cells were granulocytes ($CD15^+$); however, monocytes/macrophages ($CD14^+$) and T-cells ($CD3^+$) were also clearly detectable. B-cells ($CD19^+$) were scarcely present (Table 1).

The lymphocyte subpopulations determined in the urine, based on analysis of cells in the lymphocyte gate,[15] after five or more BCG-instillations are

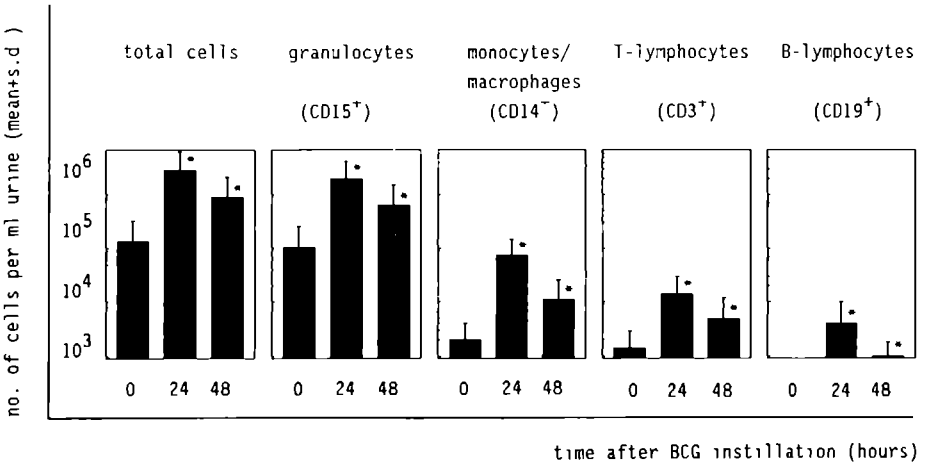

Figure 1. Increase in the total number of cells and in leucocyte subpopulations in urine of 7 patients after five and more BCG-instillations, $p<0.02$ Wilcoxon matched-pairs signed-rank test.

Table 1. Percentages of cells in leucocyte subpopulations in urine after repeated intravesical BCG-instillations.

Cell type		Cell composition (%) after [a]		
		0	24h	48h
Granulocytes[b]	CD15+	61 ± 20[c] (7)	75 ± 11 (8)	62 ± 14 (7)
Monocytes/macrophages[b]	CD14+	5 ± 6 (7)	4 ± 2 (8)	4 ± 4 (7)
T-lymphocytes[d]	CD3+	4 ± 6 (5)	1 ± 1 (5)	1 ± 1 (5)
B-lymphocytes[d]	CD19+	1 ± 1 (5)	0 ± 0 (5)	0 ± 0 (5)

[a] Time before (t=0) or after the fifth or more BCG-instillation.
[b] Determined by flow-cytofluorometric analysis of the total cell population with FSC 200.
[c] Percentage of total number of cells; mean ± s.d. of (n) patients.
[d] Determined by flow-cytofluorometric analysis of the cells in the lymphocyte gate.

presented in Table 2. For 3 patients the lymphocytes could not be analyzed in pretreatment specimen due to the lower number of cells. The mean percentage of the cells within the lymphocyte-gate reacting with the anti-CD45 mAb was 78 ± 25%, 75 ± 17% and 74 ± 7% at T_0, T_{24} and T_{48} respectively.

Table 2. Subsets of lymphocytes[a] present in urine after repeated BCG-instillations.

cell type	CD cluster	Cell composition[b] (%) after [c]		
		0	24h	48h
T-cells	$CD3^+$	86 ± 21 (5)	73 ± 12 (8)	77 ± 13 (5)
T-helper/inducer	$CD4^+$	62 ± 9 (3)	53 ± 9 (8)	49 ± 6* (5)
T-suppressor/cytotoxic	$CD8^+$	19 ± 5 (3)	23 ± 6 (8)	19 ± 9 (5)
IL-2 receptor	$CD25^+$	3 ± 3 (4)	19 ± 11** (8)	10 ± 4* (5)
HLA-DR	–	44 ± 20 (5)	47 ± 18 (7)	62 ± 25 (5)
NK-cells	$CD16^+CD56^+$	10 ± 7 (5)	16 ± 7 (8)	15 ± 11 (5)
B-cells	$CD19^+$	19 ± 28 (5)	13 ± 17 (8)	24 ± 26 (5)
Monocytes/macrophages	$CD14^+$	4 ± 6 (5)	4 ± 2 (8)	4 ± 2 (5)
Granulocytes	$CD15^+$	9 ± 4 (5)	7 ± 6 (8)	16 ± 8 (5)
CD4/CD8-ratio		3.6± 1.2 (3)	2.4± 0.7 (8)	3.0± 1.1 (5)

[a] Flow cytofluorometric analysis of cells in the lymphocyte-gate after selective cell measurement.

[b] Positive cells expressed as percentage of the $CD45^+$ cells (mean ± s.d. of n patients). Percentages $CD45^+$ cells were 78 ± 25 % (n=5), t=0; 75 ± 17 % (n=8), t=24; 74 ± 7 %, (n=5),t=48. Percentages of positive cells were determined by substraction of a conjugate control (HLA-DR and FK32) or isotype-control (others).

[c] Time before (t=o) and after the fifth or more BCG-instillation.

* p 0.05 (student's t-test)

** p 0.02 (student's t-test)

Since with the anti-CD45 Mab all types of leucocytes are detected, the number of cells reacting with the other Mabs used were expressed as percentage of the $CD45^+$ cells (Table 2). The major lymphocytes were mostly of the $CD4^+$ (helper/inducer) phenotype.

At the T_{48} the mean percentage of $CD4^+$ cells was significantly lower than in pre-instillation samples (49 ± 6% vs 62 ± 9% respectively).

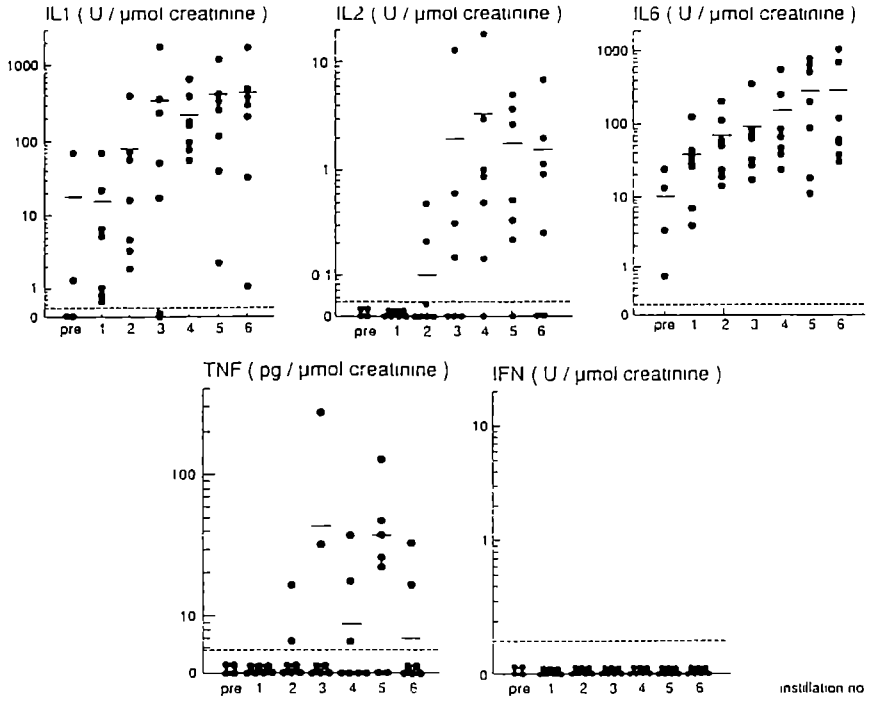

Figure 2. Cytokine levels in urine during six consecutive BCG-instillations once a week. Data presented are, for individual patients, the cytokine concentrations determined pre-treatment, i.e. before the first BCG-instillation (pre) and the highest cytokine concentration measured per instillation. • cytokine concentration in urine of 7 individual patients (pre, 4 patients). _ mean. — minimal detection limit.

In all urine specimens investigated, the percentages of $CD8^+$-cells was lower than the percentage of $CD4^+$-cells ($CD8^+$ 19-23% and $CD4^+$ 49-62%). The CD4/CD8-ratio showed a tendency to be decreased after 24h, when compared to pre-instillation values (2.4 ± 0.7 vs 3.6 ± 1.2 respectively).

HLA-DR expression was observed in a considerable percentage of the lymphocytes (44-62%) at all time points (Table 2). The percentage of lymphocytes with expression of the IL2-receptor (CD25) was significantly increased both 24h and 48h after instillation of BCG (19 ± 11% at T_{24} and 10 ± 4% at T_{48} vs 3 ±3% at T_0, $p \geqslant 0.05$ and $p \leqslant 0.05$ and $p < 0.02$ respectively). The mean percentage of NK-cells ($CD16^+$ and/or $CD56^+$) was 10-16%. However, since granulocytes may also express the CD16 antigen (Fc receptor), the real percentage of NK-cells may be lower. $CD16^+$ and/or $CD56^+$ were generally CD3. The percentage of B-cells ($CD19^+$) was 13-24%.

As is shown in Figure 1, the total number of cells was markedly increased after five or more BCG-instillations. Thus the absolute numbers of all the lymphocytes subsets, as shown in Table 2 , were increased after BCG-instillation.

Cytokines in the urine

To study cytokines in the urine of BCG treated patients, urine collected from 7 patients could be analysed. In Figure 2 the concentrations of the cytokines IL1, IL2, IL6, TNFα and IFNγ in the urine collected before (pre-treatment) and during a 6-week treatment course for individual patients are shown. In generally the cytokine concentrations in the pre-treatment samples were low or zero, only for IL1 and IL6 occasionally considerable levels were detected. During treatment the highest concentration for each instillation number measured during the first 24h after the instillation is presented.

During the first three BCG-instillations the concentration of IL1 increased markedly, reaching a plateau after 3 instillations. IL2 was not detected after the first BCG-instillation, but for the second instillation and onwards the mean IL2 concentration increased rapidly. With respect to IL6, patients already had relatively high levels in the urine after the first BCG-instillation. A moderate increase of the IL6 concentration was observed during the consecutive weeks.

Like IL2, TNFα was only detected after repeated BCG-instillations. The total number of samples positive for TNFα was low. In the urine of all of the 7 patients investigated, positive concentrations of IL1, IL2 and IL6 were measured at least once during the 6 weeks. However, for urinary TNFα 2 of the 7 patients remained negative during the complete 6-week instillation course. IFNγ could not be demonstrated. The mean concentrations of IL1, IL2, IL6 and TNFα in urine collected at regular time intervals during the first 24h after the BCG-instillations are presented in Figure 3. For this data, urine samples after the instillations 4 to 6 are used. TNFα, IL2 and IL6 were clearly detecTable by 2 hours after the instillation. In contrast, IL1 seemed to occur later, i.e. from 4 hours onwards. TNFα decreased most rapidly; it was nearly absent in 6 hour samples. Generally, IL2 was no longer detectable in the 8 hour samples, whereas IL1 and IL6 were presented up to 8 hours after instillation of BCG. The bioassay used to measure IL1 did not discriminate between IL1α and IL1β. In neutralization experiments with anti-IL1α and anti-IL1β antibodies, we observed that inhibition of the biological IL1 activity in the urine was mainly exerted by the anti-IL1α Mab, which indicates that most of the IL1 induced by the BCG-treatment is IL1α. (Figure 4)

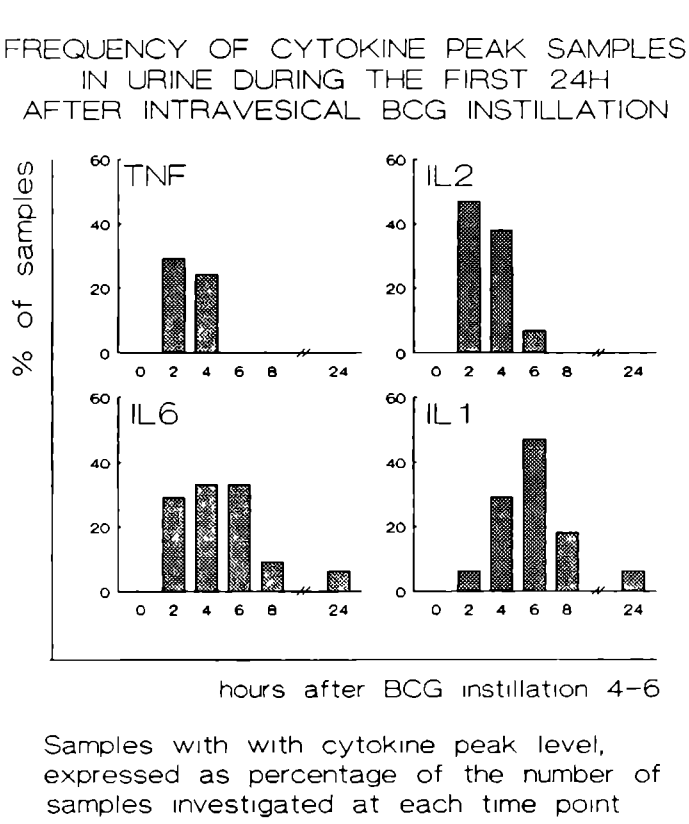

Figure 3. Occurrence of cytokine peak levels in urine during the first 24 hours after BCG-instillation 4-6. Peak level = highest cytokine concentration of the 24 hours after an instillation, evaluated per patient. Frequency of peak levels expressed as percentage of the number of samples of all patients investigated at each time point.

Additionally, cytokine concentrations were determined in urine of 2 patients intravesically treated with Mitomycin-C, a chemotherapeutic agent. Non of these specimens, obtained before and at several time points after the 6th Mitomycin-C-instillation, were positive for any of the cytokines.

DISCUSSION

The presence of leucocytes and cytokines in urine of BCG-treated patients with superficial bladder cancer indicates immunological stimulation by intravesical BCG. T-lymphocytes seem to play a crucial role in the antitumor activity of BCG.[11,12] The main cell type reported to be induced by BCG in the bladder wall of patients is the T-lymphocyte.[9] The major type of lymphocytes

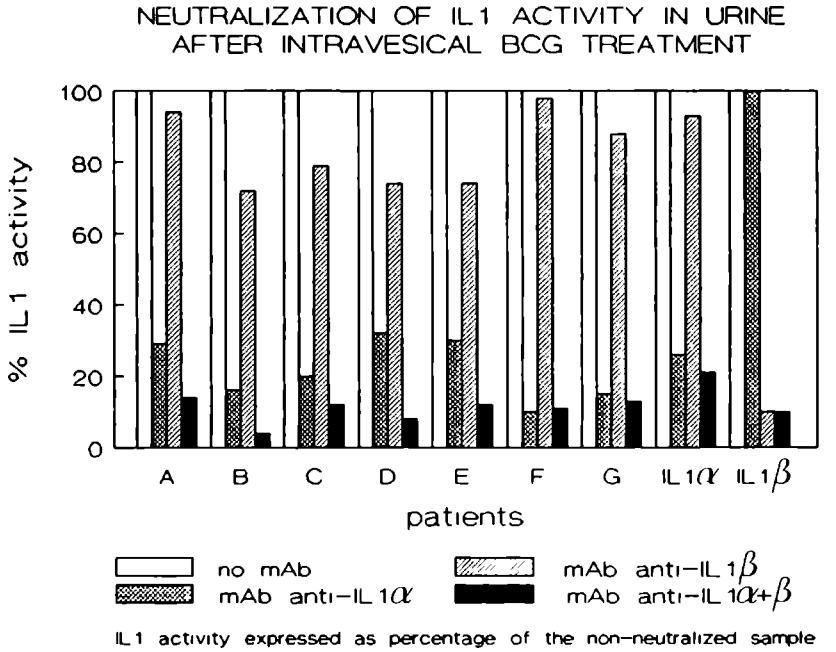

Figure 4. Neutralization of IL1 activity present in urine after intravesical BCG-treatment.

found in urine specimens were also T-cells ($CD3^+$). The majority of T-cells were $CD4^+$ (helper/inducer), whereas $CD8^+$ (suppressor/cytotoxic) T-lymphocytes were less present. The high CD4/CD8-ratios calculated and the relatively low numbers of NK-cells and B-lymphocytes (Table 2) are in agreements with the observations in biopsies and bladder washings after BCG-administration.[9,16] This suggests that the lymphocytes in urine may be a representation of the lymphocytes in the bladder wall after local administration of BCG. Expression of HLA-DR and IL2-receptors indicate activation of the T-lymphocytes by intravesical BCG-treatment.

Interleukin-2 is known to be produced by T-lymphocytes.[17] The presence of T-cells only after repeated BCG-instillations is possibly related to the presence of IL2 which was also observed only after repeated BCG-instillations.

These observations may indicate the induction of a T-cell mediated immune reaction. IFNγ is also a product of activated T-cells.[18] IFNγ is unstable in urine,[19] which may be the reason that we did not detect this cytokine.

Monocytes / macrophages are generally considered as potential producers

of IL1, IL6 and TNFα.[20,21,22] The IL1, IL6 and TNFα in the urine may be produced by macrophages, as these cells can be directly stimulated by BCG or mycobacterial antigens resulting in the release of these cytokines.[23,24,25] The fact that the type of IL1 found in the urine after BCG-treatment was mainly IL1α, is in agreement with our in vitro studies on IL1 production by human monocytes (L.A. Aarden, unpublished data). To date, the release of IL1, IL6 and TNFα has been reported for a number of other different cell types, like T-cells, endothelial cells, fibroblasts, epithelial cells or polymorphonuclear granulocytes.[20,21,22] This means that at least partly production by other cell types cannot be excluded.

Our data on the time of presence of the cytokines are generally comparable to results of other studies.[26,27,29] The increase of the concentration of all cytokines, which was found during the 6-week instillation course, may reflect an increase in the number of leucocytes infiltrating the bladder wall. For monitoring purposes, we suggest collection of urine during the first 6h after BCG-instillations four to six, since at these times the highest concentrations of important cytokines were observed.

The rationale of the BCG-treatment is to activate the immune system, ending up in an immunological reaction against locally present bladder tumors cells and tumor cell degradation.[7] Our investigations indicate a local recruitment of leucocytes which are potentially cytotoxic to tumor cells, like cytotoxic T-cells, NK-cells, monocytes/macrophages and granulocytes.[30,31,32,33]

The induced cytokines may play a role in activation of these leucocytes.[17,20,21,22,24] Besides immunomodulating properties, the cytokines themselves may have direct cytotoxic/cytostatic effects on tumor cells.[35,36,37,38] As the actual effector mechanism of BCG against superficial bladder cancer has not yet been elucidated, functional investigation of these leucocytes is warranted. Furthermore, the presence or absence of cytokines may correlate with the individual prognosis of the patients with superficial bladder cancer.[39]

CONCLUSION

The clear increase in the number of granulocytes, monocytes/macropges and T-lymphocytes which were observed in the urine 24 hours after BCG-instillations, indicate local activation of the immune system. The antigen expression of the lymphocytes suggested that they may represent the lymphocytes in the bladder wall.

These leukocytes may be usefull for functional studies, which are essential to elucidate the actual effector mechanism(s) in the mode of action of BCG against superficial bladder cancer in man.

The detection in the collected urine samples of IL2 are considered as the results of activation of BCG-specific T-cells. The presence of IL1, IL6 and TNFα might suggest activation of macrophages.

The combination of the observed leucocytes and cytokines may play an important role in the antitumor activity of BCG against bladder cancer. The presence or absence of cytokines may also have prognostic value concerning the clinical response on BCG-therapy.

The analysis of immunological products in the urine of superficial bladder cancer patients after intravesical immunotherapy with bacillus Calmette-Guérin contribute to understand the actual effects of the antitumor activity of BCG.

REFERENCES

1. Mitchell MS, Murahata RI (1979): Modulation of immunity by Bacillus CalmetteGuérin. (BCG). Pharmac Ther 4:329-353.

2. De Jong WH, Steerenberg PA, Ruitenberg EJ (1987): Bacillus Calmette Guérin (BCG) and its use for cancer immunotherapy. In: Den Otter W and Ruitenberg E (eds), Tumor Immunology Mechanisms, Diagnosis, Therapy. Amsterdam, Elsevier, pp. 283-307.

3. Witjes JA, Meijden APM van der, Debruyne FMJ (1990): Use of intravesical Bacillus Calmette-Guérin in the treatment of superficial transitional cell carcinoma of the bladder: an overview. Urol.Int. 45:129-136.

4. Herr HW, Laudone VP, Badalament RA (1988): Bacillus Calmette-Guérin therapy alters the progression of superficial bladder cancer. J.Clin.Oncol. 6:1450-1455.

5. Paulie S, Perlman P (1987): Immunological features of human bladder cancer. In: Byers VS and Baldwin RW (eds): Immunology of malignant disease. Lancaster, MTP Press Limited, pp. 151 166.

6. Haas GP, Saloman D, Rosenberg SA (1990): Tumor-infiltrating lymphocytes from nonrenal urological malignancies. Cancer Immunol.Immunother. 30:32-35.

7. Ratliff TL (1989): Mechanisms of action of BCG for bladder cancer. Progr. Clin.Biol. Res. 310: 107-12.

8. Torrence RJ, Kavoussi LR, Catalone WJ, Ratliff TL (1988): Prognostic factors in patients treated with intravesical Bacillus Calmette-Guérin for superficial bladder cancer. J.Urol. 139:941-944.

9. El-Demiry MIM, Smith G, Ritchie AWS, James K, Cumming JA, Hargreave TB, Chisholm GD (1987): Local immune response after intravesical BCG-treatment of carcinoma in situ. Br.J.Urol. 60:543 548.

10. Haaff EO, Dresner SM, Kelley DR, Ratliff TL, Shapiro A, Catalone WJ (1985): Role of immunotherapy in the prevention of recurrence and invasion of urothelial bladder tumors: a review. World J.Urol.3:76-85.

11. Hanna MGR, Snodgrass MJ, Zbar B, Rapp HJ (1973): Histopathology of tumor regression after intralesional injection of Mycobacterium bovis: Development of immunity to tumor cells and BCG. J.Nath.Canc.Inst.51:1897-1908.

12. Ratliff TL, Gillen DP, Catalona WJ (1987): Requirement of a thymus dependent immun response for BCG-mediated antitumor activity. J Urol.137:155-157.

13. Meijden APM van der, De Jong WH, Steerenberg PA, Walvoort HC, DeBoer EC, Debruyne FMJ, Ruitenberg EJ: Intravesical BCG-administration in the guinea pig. A histomorphological study. Virchows. (Cell.Pathol.)55; 207-215.

14. De Jong WH, De Boer EC, Meijden APM van der, Steerenberg PA, Debruyne FMJ (1991): Intravesical BCG-administration in the guinea pig II. Immunohistochemical characterization of cellular infiltrate after one or two cycles of intravesical BCG. Virchows Arch (B).

15. De Boer EC, De Jong WH, Meijden APM van der, Steerenberg PA, Witjes JA, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1991): Leucocytes in the urine after intravesical BCG-treatment for superficial bladder cancer· a flow cytofluorometric analysis. Urol.Res.19:45-50.

16. Weiner RS, Linn R, Wasjman Z, Hackett RI (1988): Identification of T subpopulation in human bladder washing during a six week course of intravesical BCG. J.Urol. 141: 291A (abstr 487).

17. Watson J, Mochizuki D, Gillis S (1988): T-cell growth factors: Interleukin2. Immunol. Today 1:113-136.

18. Trinchiere G, Persussia B (1988): Immune interferon: a pleiotropic lymphokine with multiple effects. Immunol.Today 6:131-136.

19. Prescott S, James K, Hargreave TB, Chisholm GD, Smyth JF (1988): Radioimmunoassay detection of interferon-gamma in urine after intravesical Evans BCG-therapy. J. Urol. 144:1248-1251.

20. Dinarello CA (1989): Interleukin-1 and its biological related cytokines. Adv. Immunol .44:153-205.

21. Kishimoto T (1989): The biology of interleukin-6. Blood 74:1-10.

22. Semenzato G (1990): Tumor necrosis factor:a cytokine with multiple biological activities. Br.J.Cancer 61:354-361.

23. Sanceau J, Falcoff R, Beranger F, Carter DB, Witzerbin (1990): Secretion of interleukin-6 (IL-6) by human monocytes stimulated by muramyl dipeptide and tumor necrosis factor alpha. Immunology 69:52-56.

24. Wallis RS, Fujiwara H, Ellner JJ (1989): Direct stimulation of monocyte release of interleukin1 by mycobacteria protein antigens. J.Immunol. 136:193-196.

25. Valone SE, Rich EA, Wallis RS, Ellner JJ (1988): Expression of tumor necrosis factor in vitro by human mononuclear phagocytes stimulated with whole Mycobacterium bovis BCG and mycobacterial antigens. Infect Immun. 56:3313-3315.

26. De Jong WH, De Boer EC, Meijden APM van der, Vegt PDJ, Steerenberg PA, Debruyne FMJ, Ruitenberg EJ (1990): Presence of interleukin-2 in urine after intravesical treatment of superficial bladder cancer patients with Bacillus Calmette-Guérin. Cancer Immunol.Immunother.31 182-186.

27. Ratliff TL, Haaff EO, Catalona WJ (1986): Detection of interleukin2 in the urine of patients with superficial bladder tumors after treatment with intrave sical BCG. Clin.Immunol.Immunopathol.40:375-379.

28. Bohle A, Nowe C, Ulmer AL, Musehold J, Gerdes J, Hofstetter AG, Flad HD (1990): Detection of urinary TNF,IL1 and IL2 after local BCG-immunotherapy for bladdercarcinoma. Cytokine 2:175-181.

29. Schamhart DHJ, Kurth K (1990): Detection of urinary IL6 during intravesical BCG-therapy. J.Urol. 143:321A (abstr 531).

30. Van Kessel KPM, Verhoef J (1990): A view to a kill: Cytotoxic mehanisms of human polymorphonuclear compared with monocytes and natural killer cells. Pathobiol.58:249-264.

31. Herberman RB, Ortaldo JR (1990): Natural killer cells:their role in defence against autologous human tumors. Immunol.Rev.116:33-61

32. Kleinerman ES, Herberman RB (1984): Tumoricidal activity of human monocytes: evidence for cytolytic function distinct from that of NK-cells. J.Immunol.133:4-6.

33. Mukherji B, Charaborty NG, Sivanandham M (1990): T-cell clones that react against autologous human tumors. Immunol.Rev.116:33-61.

34. Herberman RB (1987): Lymphokine-activated killer cell activity. Immunol. Today 8:178-181.

35. Chen L, Mory Y, Zilberstein A, Revel M (1988): Growth inhibition of human breast carcinoma and leukaemia/lymphoma cell lines by recombinant interferon-beta. Proc.Natl. Acad.Sci.USA 85:8037-8041.

36. Endo Y, Matsushima K, Oppenheim JJ (1986): Mechanisms of in vitro antitumor effects of interleukin1 (IL1). Immunobiology 172:316-322.

37. Ruggiero V, Latham K, Baglioni C (1987): Cytostatic and cytotoxic activity of tumor necrosis factor on human cancer cells. J.Immunol.138:2711-2716.

38. Stotter H, Wiebke EA, Tomita S, Belldegrum A, Topalian S, Rosenberg SA, Lotze MT (1989): Cytokines alter target cell susceptibility to lysis II. J.Immunol.142:1767-73.

39. Reijke TM de, Boer LC de, Heinz Kurth K, Schamhart DHJ (1996): Urinary cytokines during intravesical Bacillus Calmette-Guérin therapy for superficial bladder cancer: Processing, stability and prognostic value. J.Urol. 155:477-482.

A RANDOMIZED STUDY OF INTRAVESICAL MITOMYCIN-C, BCG-TICE AND BCG-RIVM TREATMENT IN PTA-PT1 PAPILLARY CARCINOMA AND CARCINOMA IN SITU OF THE BLADDER

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INTRODUCTION TO CHAPTER 4

The primary goal of intravesical chemotherapy in patients with bladder cancer is to eradicate the existing disease (treatment) and/or to prevent recurrent disease (prophylaxis). Superficial bladder cancer, initially treated with transurethral resection has a tendency to recur and to progress and therefore intravesical prophylaxis of recurrence is essential. A minority of the patients with recurrent superficial bladder disease will eventually progress and the question remains whether intravesical chemo-immunotherapy can influence or, if possible, eventually prevent progression. It is obvious that dealing with carcinoma in situ, a superficial bladder cancer not amendable by transurethral resection, intravesical therapy has a therapeutic objective, which can be evaluated by cytology.

Differences in therapeutic efficacy and toxicity of intravesical therapy with immunological agents can be evaluated in prospective randomized trials. Clinical prospective research is indeed essential to underline advantages and disadvantages of one substance over another. Moreover, it is essential to compare the efficacy and value of intravesical immunotherapy with intravesical chemotherapy. For this reason the Dutch South-East Cooperative Urological Group has executed different trials, comparing intravesical immunotherapy and chemotherapy, and this chapter deals with a large randomized trial comparing intravesical Mitomycin-C therapy with BCG-Tice and BCG-RIVM in the treatment of pTa-pT1 papillary carcinoma and carcinoma in situ of the urinary bladder.

This trail started in 1987 and was closed for inclusion as of 1991. The first results of this trial with respect to toxicity, local and systemic side-effects has been reported in 1993 by Witjes et al..[1] This publication also described a preliminary (lack of) difference between the three treatment arms.

Maturity of the data and results of a clinical trial are usually obtained when at least 50% of the patients have shown a recurrent tumor. After two years of followup, as published by Witjes et al , the recurrence rate in the Mitomycin group was 35%, in the BCG-Tice group it was 46% and in the BCG-RIMV group it was 38% respectively. An updated evaluation in 1995, as described in this paper, did not completely reach the maturity as defined above, but the recurrence rates were still less than 50%, namely 43% in Mitomycin-C, treated patients, 64%in the BCG-Tice group and 46% in the

BCG-RIMV group. However, these percentages show that close to 50% of the patients had experienced a recurrence.

It is remarkable that the increase in recurrence rate between 2 and 5 years is only small and limited and hence it is difficult to estimate when exactly the 50% recurrence rate in all three arms will be reached. This is (partially) due to the selection criteria used in the trail, which also included patients with low risk of recurrence. This is one of the flaws of this trail as described in the discussion of chapter 4.

The progression rate in patients with superficial bladder cancer is low. A former study analyzing the available clinical outcome data within the Dutch South-East Cooperative Urological Group (Kiemeney et al.[2]) have calculated the actual risk of disease progression for primary superficial bladder cancer into at least pT2 tumors as 10.2% after 2 years with hardly any increase after 5 years till 13.3%. This is somewhat lower than progression rates usually described in literature (+/- 20%) but the latter is an estimation rather than a fact.

It also depends on the initial stage (pTa or pT1) and grade (grade I to 3 +/- carcinoma in situ) of the tumors described in the different series. In view of the low recurrence rate, it is difficult to estimate or calculate the influence of intravesical therapy on progression. However, this is an important fact since the value of intravesical therapy would increase tremendously when it could be proven that it influences progression. So far this has not been observed in the study described in chapter 4 and has not been described in other randomized trails except for an anticipating calculation as described by Lamm et al..[3] However, the assumption of Lamm is a statistical consideration rather than an outcome of a clinical trial. To evaluate the influence on progression of intravesical therapy, another study design is needed with higher numbers of patients having tumors with a higher risk of recurrence and progression and with a (very) long followup. This has not been the case in any of the studies published so far and it is also not possible to analyze this in the study described hereafter. However, the value of the described study is, that it did analyze in detail the effect on superficial bladder recurrence which is an objective trial endpoint and can be considered as treatment failure.

REFERENCES

1. Witjes JA, Meijden APM van der, Witjes WPJ, Doesburg W, Schaafsma HE, Debruyne FMJ and members of the Dutch South-East Cooperative Urological Group (1993): A randomized prospective study comparing intravesical instillations of Mitomycin-C, BCG-Tice and BCG-RIVM in pTa-pT1 tumours and primary carcinoma in situ of the urinary bladder. Eur.J. Cancer 29A:1672-1676.

2. Kiemeney LALM, Witjes JA, Verbeek ALM, Heijbroek RP, Debruyne FMJ and the members of the Dutch South-East Cooperative Urological Group (1993): The clinical epidemiology of superficial bladder cancer. British J. Cancer 67:806-812.

3. Lamm DL (1992): Long-term results of intravesical therapy for superficial bladder cancer. Urol.Clin.North.Amer. 19-3:573-580.

ABSTRACT

Results of a randomized prospective study are reported in which Mitomycin-C, Bacillus Calmette-Guérin (BCG)-Tice strain and BCG-RIVM strain are compared in 437 patients with primary or recurrent pTa and pT1 bladder tumors, including carcinoma in situ. The followup (= time in study) varied from 2 till 81 months (mean 36 months). After complete transurethral resection of all visible tumors the patients have been treated with Mitomycin-C (30 mg once a week for 4 consecutive weeks and thereafter every month for a total of 6 months), BCG-Tice or BCG-RIVM (5×10^8 colony forming units once a week for 6 consecutive weeks).

For papillary tumors the Mitomycin-C and BCG-RIVM treatments were equally effective ($p=0.53$). Mitomycin-C was more effective than BCG-Tice therapy ($p=0.01$).

INTRODUCTION

In pTa and pT1 bladder cancer both intravesical chemotherapy and immunotherapy are used as adjuvant treatment after transurethral resection. Both therapies can reduce the high rate of recurrences in papillary tumors as well as eradicate carcinoma in situ.[1,2,3,4,5] Response rates of different studies comparing chemotherapeutical agents versus BCG after transurethal resection in patients with pTa, pT1 and carcinoma in situ bladder tumors suggests that BCG is superior to Thiotepa, Doxorubicin or Mitomycin-C.[4,6]

Mitomycin-C was selected as a chemotherapeutic agent in this study because of its proved efficacy in regard to prevention of tumor recurrence and high response rate in patients with carcinoma in situ.[7,8,9]

In 1988 we reported the results of a prospective trial in which Mitomycin-C was compared with BCG-RIVM, and no statistically significant differences in toxicity and efficacy were noted between the two arms.[10] Because of the lack of superiority of BCG-RIVM over Mitomycin-C, the efficacy of BCG-RIVM was questioned. BCG is a biological product, consisting of living bacteria, subcellular debris and adjuvant compounds. Considerable differences in the various strains and even in lots of the same strain can be present.[11] Most of the BCG-strains, e.g. BCG-Tice, are grown as a surface pellicle, ground in a ball mill, resuspended and freeze dried. BCG-RIVM, however, is grown in a homogeneously dispersed culture and lyophilized in a solution containing glucose and tween 80.

It has been suggested that those strains with the best ability to bind to fibronectin could be the strains with the most potent antitumor efficacy. The glycoprotein fibronectin has an important role in the attachment of BCG to the bladder wall, which has been demonstrated in mice.[12] At least in the mouse model BCG-strains grown as a surface culture (Tice) showed a better attachment to the bladder wall than homogeneously dispersed cultured BCG (RIVM).[13]

These observations led to the necessity to compare the two different BCG-strains to each other and to a well known chemotherapeutic drug.

Objectives of the study

The first objective of this three-arm prospective, controlled and randomized trial was to compare the efficacy of Mitomycin-C chemotherapy with that of BCG-immunotherapy, using two different substrains in patients with primary or recurrent pTa, pT1 and carcinoma in situ bladder tumors. The parameters compared were duration of disease-free interval and the rate of progression

to a higher stage (T category) of disease.

The second objective was to compare the incidence and severity of side effects of the treatments.

Primary endpoint of the study was the time to recurrence of bladder tumors, which was considered treatment failure.

MATERIALS AND METHODS

From april 1987 to december 1990, 469 patients entered the study from 27 institutions of the Dutch South East Cooperative Urological Group. All patients had histologically proved papillary pTa-pT1 transitional cell carcinoma of the bladder with or without concomitant or primary carcinoma in situ. Pathological classification was done according to the TNM system,[14] and grading of the tumors was done according to the method of Koss.[15] The highest stage and grade of all tumor specimens in individual patients were used to characterize each patient. All specimens of tumor and bladder biopsies were reviewed by a single pathologist.

The toxicity, local and systemic side effects, has been reported elsewhere.[16] Briefly, side effects were divided as local, systemic or allergic. Local toxicity was defined as the occurrence of culture proved bacterial cystitis (not BCG related), drug-induced cystitis (Mitomycin-C or BCG related) or other local side effects, such as hematuria, prostatitis and epididymitis. The severity of side effects was scored by classifying them as requiring no delay, delay or cessation of instillation therapy.

Treatment

Intravesical chemotherapy consisted of 30 mg Mitomycin-C in 50 ml saline instilled once a week for 1 month (week 1 to 4) and thereafter once a month for a total of 6 months. If superficial tumor recurred or carcinoma in situ persisted at 3 months the treatment schedule was not changed after complete transurethal resection (or biopsy) and if disease recurred or persisted after 6 months 3 additional monthly Mitomycin-C instillations were given.

Intravesical immunotherapy was performed with BCG-Tice or with BCG-RIVM. BCG was administered (5×10^8 colony forming units in 50 ml saline) once a week for 6 consecutive weeks. If superficial tumor recurred or carcinoma in situ persisted at 3 or 6 months a second 6-week course with BCG-instillations was given after complete transurethral resection or biopsy. In all other cases of recurrences, irrespective of the treatment arm, the patient went off study and treatment was left to the discretion of the urologist.

Followup and treatment response

Followup cystoscopy was performed at 3-month intervals during the first 2 years after transurethal resection, and thereafter at 4 and 6-month intervals. Cytology was done at each cystoscopy.

For papillary tumors the efficacy of the therapy was evaluated by determining the disease-free period after randomisation.

The response to therapy in patients with carcinoma in situ was scored as no response or a complete response. Complete response was defined as the complete disappearance of carcinoma in situ, documented by normal urine cytology, cystoscopic examination and bladder biopsies.

Statistical analysis

To detect a difference of 50% in the median duration of the disease-free interval between Mitomycin-C and the best (smallest hazard rate) of the BCG-treatments (assuming the time to recurrence follows an exponential distribution), 90 eligible and evaluable patients followed until recurrence were required in each treatment arm (error probabilities $\alpha = 0.05$ and $\beta = 0.20$). Assuming that 65% of the patients will have at least one recurrence during the study, 138 evaluable patients per treatment arm were needed, for a total of 414 evaluable patients. For randomization the restricted blockwise (blocksize 6 equals 3 treatments times 2 patients per treatment) randomisation was used. Estimation of the cumulative distribution of the disease-free interval was performed by the Kaplan-Meier method for each of the three treatment groups and for subgroups. Differences in the distribution of the disease-free interval were tested with the log rank test. For testing of the difference between sample percentages the chi-square test was used. All statistical computations were done using Statistical Analysis System procedures

RESULTS

Eligibility

Of the 469 patients who entered the study, 17 patients (6%) were ineligible because of no malignancy after pathological review (n=9), tumor stage greater than pT1 after pathological review (n=6) and prior intravesical chemotherapy (n=2), and they were excluded from subsequent analysis. Another 15 patients (3%) were also excluded from subsequent analysis because of first instillation more than 4 weeks after transurethal resection (n=4), no instillations started

due to hematuria (n=2), refusal (n=2), medication problems (n=2), death (n=1), myocardial infarction (n=1) before the instillation procedure and other reasons (n=3). Ultimately 437 patients were eligible for analysis. Followup (time in study) of these patients varied from 2 to 81 months (mean 36 months).

Patient and tumor characteristics

Tumor characteristics were equally distributed among the 3 treatment arms (Table 1). Of the 437 eligible patients, 50 had carcinoma in situ, 254 had pTa tumors and 133 patients had pT1 tumors (Table 1). Of the patients with carcinoma in situ, 12 received Mitomycin-C, 23 received BCG-Tice and 15 BCG-RIVM. After complete transurethral resection patients with pTa or pT1 papillary tumors were treated with Mitomycin-C (136), Tice-BCG (117) and RIVM-BCG (134). Of the patients with carcinoma in situ 25 patients had pure flat multifocal carcinoma in situ and 25 also had papillary tumors (concomitant carcinoma in situ). Of all patients 84 had pTaG1 tumors, including 43 patients with a solitary tumor and 41 patients with multiple (2-8) tumors.

Off-study patients

A total of 230 patients (74 Mitomycin-C, 76 BCG-Tice, 80 BCG-RIVM) went off study because of recurrence at or after 9 months (122), protocol violation (36), progression in tumor stage to T2 or higher (23), adverse effects (14), intercurrent death (10), treatment refusal (7), lost to followup (3) and other reasons (15). A detailed differentiation of reasons for off study is shown in Table 2.

Efficacy of treatment

For stages pTa and pT1 papillary tumors the time to first recurrence was recorded. Recurrence was noted during the study in 58 of 136 patients (43%) treated with Mitomycin-C, 75 of 117 (64%) treated with Tice-BCG and 62 of 134 (46%) treated with RIVM-BCG. The estimated percentages of disease-free patients in the 3 treatment arms are shown in Table 3 The analysis of efficacy in the patients with papillary tumors shows a statistically significant difference among the treatment arms (p=0.04). The Mitomycin-C and BCG-RIVM treatments were equally effective (p=0.53), but Mitomycin-C treatment was more effective than BCG-Tice (p=0 01), see Figure 1.

For carcinoma in situ tumors the complete response was analyzed as parameter of efficacy. In all patients with carcinoma in situ the complete

Table 1. Tumor characteristics of evaluable patients and allocation to treatment.

	Mitomycin-C (148)	BCG-Tice (140)	BCG-RIVM (149)	Total (437)
G1	24	28	32	84
pTa G2	55	48	54	157
G3	4	5	4	13
				254 (65.6%)
G1	–	–	–	–
pT1 G2	31	18	23	72
G3	22	18	21	61
				133 (34.4%)
primary	104	87	108	299
recurrent	32	30	26	88
total pap.tumors	136	117	134	387
carcinoma in situ	12	23	15	50

Table 2. Reasons off study differentiated by treatment arm.

	Mitomycin-C		BCG-Tice		BCG-RIVM		Total	
	no-cis*	cis**	no-cis*	cis**	no-cis*	cis**	no-cis*	cis**
Recurrences	31	3	39	7	36	6	106	16
Protocol violation	14	6	8	1	6	1	28	8
Progression	8	–	5	2	4	4	17	6
Adverse effect	3	–	5	2	4	–	12	2
Death	2	–	1	2	4	1	7	3
Refusal	1	–	1	–	5	–	7	–
Lost to followup	1	–	1	–	1	–	3	–
Other	5	–	2	–	6	2	13	2
TOTALS	65	9	62	14	66	14	193	37

* no carcinoma in situ; **carcinoma in situ

response was 68% for a duration of 2 till 49 months (mean 19). Complete response was 67% (8 of 12 patients) for Mitomycin-C, 74% (17 of 23) for Tice BCG and 60% (9 of 15) for RIVM-BCG. These results do not allow any conclusion to be drawn because the numbers of patients randomized are too small.

Table 3. Treatment efficacy, defined as percentage of patients free of tumor at 5 years of followup. % disease-free with standard error, *all* papillary tumors.

	Mitomycin-C	BCG-Tice	BCG-RIVM
1 year	73±4	66±5	70±4
2 years	61±5	53±5	61±5
3 years	59±5	42±5	55±5
4 years	57±5	36±5	54±5
5 years	57±5	36±5	54±5*

* no failures seen after 4 years

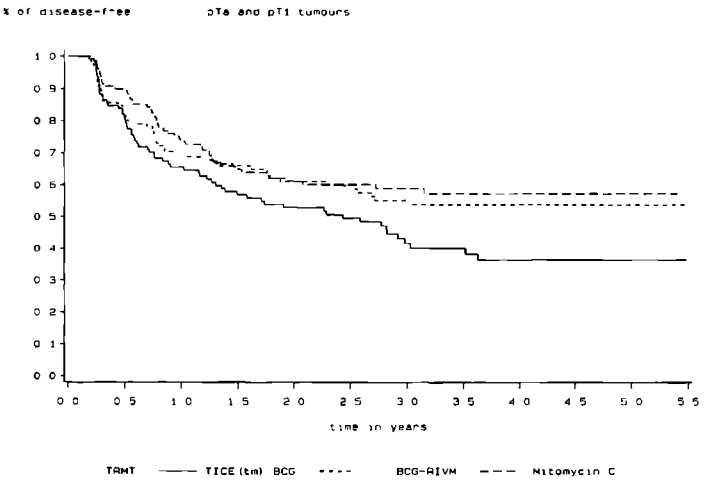

Figure 1. Kaplan-Meier plots of the percentage of patients with papillary tumors pTa and pT1, grade 1-3 transitional cell cancer, free of tumor after transurethal resection and treatment with Mitomycin-C (dashed line), BCG-Tice (solid line) and BCG-RIVM (dotted line). Log-rank test: p=0.04.

Progression in tumor stage

Progression in stage (pT2 or higher) at recurrence (patients who went off study) was observed in 8 (6%) patients of the Mitomycin-C-group, 7 (5%) of the BCG-Tice group and in 8 (6%) treated with the BCG-RIVM.

Toxicity

The number and severity of adverse effects were recorded during the instillation period (6 months for Mitomycin-C, 6 weeks for BCG) and are listed in Table 4. Between the BCG groups no statistical difference in number and severity was found. Drug-induced cystitis, other local side effects and systemic side effects were significantly less frequent in the Mitomycin-C group. Three of 148 patients (2%) had to stop Mitomycin-C treatment, 5 of 140 (4%) stopped Tice BCG and 11 of 149 (7%) stopped RIVM-BCG. No life threatening adverse effects or treatment related deaths were recorded.

DISCUSSION

The primary objective in this study was to compare the efficacy of Mitomycin-C chemotherapy with that of BCG-immunotherapy, using 2 different strains, in patients with pTa, pT1 and carcinoma in situ bladder cancer. The efficacy in patients with papillary tumors was related to the percentage disease-free period after transurethal resection of all visible tumors. When all patients with papillary tumors (grades 1, 2 and 3 and stages pTa and pT1) were analyzed together a statistical significant difference was seen in efficacy among the 3 treatment schemes in favor of the Mitomycin-C and BCG-RIVM treated patients when compared to BCG-Tice treated patients.

Because of statistical reasons, definitive results of this study should be withheld until at least 50% of patients in each of the 3 arms have shown recurrent tumor. However, after 3 years of followup only the BCG-Tice group has a slight decrease from 42% disease-free at 3 years to 36% after 4 and 5 years, respectively (Table 2). The disease-free percentage for Mitomycin-C decreases only from 59% at 3 years to 57% at 5 years and for BCG-RIVM it decreases only from 55% to 54% at 3 and 5 years, respectively. Since in superficial bladder studies most recurrences are observed in the first 2 years after randomization, it is hard to estimate how many years are needed to obtain less than 50% disease-free in all 3 arms (see fig.).

Only a few studies have presented a 5 year followup of patients with superficial bladder cancer treated with Mitomycin-C or BCG. Lamm[17] reported the estimated 5-years recurrence rates of 78% for Mitomycin-C treated

Table 4 Side effects during instillation period

	Mitomycin-C	BCG Tice	BCG RIVM	total
bacterial cystitis (p=0 20)				
no	121	102	115	338
yes, without delay	25	26	24	75
yes, with delay	1	11	10	22
stop treatment	1	1	–	2
total yes (%)	(18 2) **27**	(27 1) **38**	(22 8) **34**	(22 7) **99**
drug induced cystitis (p=0 009)				
no	122	98	101	321
yes, without delay	25	40	45	11
yes, with delay	1	1	2	4
stop treatment	–	1	1	2
total yes (%)	(17 6) **26**	(30 0) **42**	(32 2) **48**	(26 5) **116**
allergic reaction (p=0 30)				
no	141	137	146	424
yes, without delay	4	2	2	8
yes, with delay	1	1	1	3
stop treatment	2	–	–	2
total yes (%)	(4 7) **7**	(2 1) **3**	(2 0) **3**	(3 0) **13**
other local side effects (p=0 004)				
no	141	117	127	385
yes, without delay	7	23	20	50
yes, with delay	–	–	2	2
total yes (%)	(4 7) **7**	(16 4) **23**	(14 8) **22**	(11 9) **52**
systemic side effects (p<0 001)				
no	142	102	122	366
yes, without delay	6	32	24	62
yes, with delay	–	3	1	4
stop treatment	–	3	2	5
total yes (%)	(4 1) **6**	(27 1) **38**	(18 1) **27**	(16 3) **71**

patients and 48% for BCG treated patients. This means that 22% of patients treated with Mitomycin-C and 52% of those treated with BCG were tumor-free after 5 years. Our results are comparable for BCG patients. The percentage of patients free of tumor after 5 years was 54% of RIVM-BCG, 36% of Tice-BCG and 57% of Mitomycin-C treated patients.

Although intravesical chemotherapy delays the time to first recurrence, it is unknown whether chemotherapy influences progression and survival. Recent studies suggest that the time to progression is prolonged with intravesical immunotherapy in patients with superficial bladder cancer.[18,19] The progression rate in our study was low at 6%, 5% and 4% for Mitomycin-C, BCG-Tice and BCG-RIVM, respectively. Our data do not indicate that the progression rate to muscle-invasive disease was different in any of the treatment arms, which may explain the relatively low number of grade 3 and T1 tumors as well as the low number of carcinoma in situ patients. Most of the patients had intermediate risk tumors. Tumor grade did not significantly influence the time to treatment failure for any of the groups.

Our study reports 84 patients with pTa grade 1 tumors. These low stage, low grade tumors have a low risk for recurrence or progression if they are solitary. Of the 84 pTa grade 1 patients, 43 had a solitary tumor and 41 had multiple primary or recurrent tumors. However, multiple pTa grade 1 tumors have an intermediate risk for recurrences, which indicates that in our study only 43 of all eligible patients (9.8%) had a low risk for recurrence or progression. This also means that 90.2% of the patients may have had at least potential benefit of adjuvant intravesical therapy after transurethal resection. Therefore, the significance of the results is not particularly diluted by inclusion of the 10% of patients at low risk.

Comparing a 6-week BCG schedule to 6 months of Mitomycin-C therapy, our data do not provide evidence that BCG offers better response in the treatment of patients with pTa and pT1 bladder tumors than Mitomycin-C. In patients with primary or concomitant carcinoma in situ, immunotherapy with BCG also was not superior to chemotherapy with Mitomycin-C. However, the efficacy results in patients with carcinoma in situ, do not allow any conclusion to be drawn because too few patients with carcinoma in situ were entered in this study.

This study does not address the issue whether a 6-week course of BCG is less effective than BCG maintenance therapy. However, it remains useful to look at the response rates in different studies in which 6-week courses, repeated 6-week courses or maintenance therapy have been used.[4,19,20] Com-

parisons with other reported studies must be interpreted with caution because of multiple variable factors, such as different doses, instillation schedules, selected patient groups and so forth.

In Southwest Oncology Group study 8795 a randomized comparison of BCG-Tice and Mitomycin-C was performed in 469 patients.[21] Of 377 evaluable patients 37 tumor recurrences were documented in the 190 BCG treated patients (19.4%) versus 61 recurrences in the 187 Mitomycin-C treated patients (32,6%)(p=0.0052). The highly significant advantage of BCG over Mitomycine-C prompted early closure of the trial. However, in the Southwest Oncology Group trial at least 3 major differences can be noted compared to our study. In study 8795 only patients with rapidly recurring tumors (high risk) were enrolled, they were treated by maintenance therapy and the 20 mg dose of Mitomycine-C was low.

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REFERENCES

1. Witjes JA, Van der Meijden APM and Debruyne FMJ (1990): Use of intravesical Bacillus Calmette-Guérin in the treatment of superficial transitional cell carcinoma of the bladder: an overview. Urol. Int. 45: 129.

2. Lamm DL, Thor DE and Radwin HM (1982): Bladder cancer immunotherapy. J. Urol. 128: 932.

3. Pinsky CM, Camacho F, Kerr D, Braun DW jr., Whitmore WF jr. and Oettgen HF (1982): Treatment of superficial bladder cancer with intravesical BCG. In: Terry WD and Rosenberg SA, eds. Immunotherapy of human cancer. New York: Expcerpta Medica: 309.

4. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, Stanisic TH, Smith JA Jr., Sullivan J, Sarosdy MF, Crissman JA and Coltman CA (1991): A randomized trial of intravesical doxorubicin and immunotherapy with bacillus Calmette-Guérin for transitional-cell carcinoma of the bladder. N. Engl. J. Med. 325: 1205.

5. DeJager R, Guinan P, Lamm D, Khanna O, Brosman S, DeKernion J, Williams R, Richardson BA, Muenz L, Reitsma D and Hanna MG jr. (1991): Long-term complete remission in bladder carcinoma in situ with intravesical Tice Bacillus Calmette-Guérin. Overview analysis of six phase II clinical trials. Urology 38: 507.

6. Herr HW (1987): Intravesical therapy. A critical overview. Urol. Clin. N. Amer. 14: 399.

7. Huland H, Otto U, Droese M and Kloeppel G (1984): Long-term mitomycin C instillation after resection of superficial bladder carcinoma: influence on recurrence, progression, and survival. J. Urol. 132: 27.

8. Bracke B, Johnson D, Von Eschenbach A, Swanson D, De Furia D and Crook S (1980): Role of intravesical mitomycin in management of superficial bladder tumors. Urology 16: 10.

9. Soloway MS (1987): Evaluation and management of patients with superficial bladder cancer. Urol. Clin. N. Amer. 14: 777.

10. Debruyne FMJ, Van der Meijden APM, Geboers ADH, Franssen MPH, Van Leeuwen MJW, Steerenberg PA, de Jong WH and Ruitenberg JJ (1988): BCG (RIVM) versus mitomycin intravesical therapy in superficial bladder cancer. First results of randomized prospective trial. Suppl. Urology 31:2.

11. Kelley DR, Ratliff TL, Catalona WJ, Shapiro A, Lage JM, Bauer WC, Haaff EO and Dresner SM (1985): Intravesical Bacillus Calmette-Guérin therapy for superficial bladder cancer: effect of Bacillus Calmette-Guérin viability on treatment results. J. Urol. 134: 48.

12. Ratliff TL, Palmer JO, McGarr JA and Brown EJ (1987): Intravesical Bacillus Calmette-Guérin therapy for murine bladder tumors: initiation of response by fibronectin-mediated attachment of Bacillus Calmette-Guérin. Cancer Res. 47: 1762.

13. Ratliff TL, Kavoussi LR, Catalone WJ (1988): Role of fibronectin in intravesical BCG-therapy for superficial bladder cancer. J.Urol. 139: 410-418.

14. Hermanek P, Sobin LH (1987): TNM classification of malignant tumors. International Union Against Cancer. Fourth, fully revised edition. Heidelberg: Springer-verlag.

15. Koss LG (1975): Tumors of the urinary bladder. Atlas of tumor pathology, 2nd series. Fascicle 11, Washington D.C.: Armed Forces Institute of Pathology.

16. Witjes JA, Van der Meijden APM, Witjes WPJ, Doesburg W, Schaafsma HE, Debruyne FMJ (1993): A randomized prospective study comparing intravesical instillations of Mitomycin-C, BCG-Tice and BCG-RIVM in pTa-pT1 tumors and primary carcinoma in situ of the urinary bladder. Eur.J.Cancer. vol. 29A. no. 12.1672.

17. Lamm DL (1992): Long-term results of intravesical therapy for superficial bladder cancer. Urol.Clin.N.Amer. 19:573.

18. Reitsma DJ, Guinan P, Lamm DL, Khanna OP, Brosman SA, DeKernion JB, Williams RD, Simpson G and Hanna MG jr. (1989): Long term effect of intravesical Bacillus Calmette-Guérin (BCG) Tice strain on flat carcinoma in situ of the bladder. In: Debruyne FMJ, Denis L and Van der Meijden APM (eds.): BCG in superficial bladder cancer. Vol. 310 of Progress in clinical and biological research. New York: Alan R. Liss.: 179.

19. Badalament RA, Herr HW, Wong GY, Gnecco C, Pinsky CM, Whitmore WF jr., Fair WR and Oetggen HF (1987): A prospective randomized trial of maintenance versus non-maintenance intravesical Bacillus Calmette-Guérin therapy of superficial bladder cancer. J. Clin. Oncol. 5: 441.

20. Hudson MA, Ratliff TL, Gillen DP, Haaff EO, Dresner SM and Catalona WJ (1987): Single course versus maintenance Bacillus Calmette-Guérin therapy for superficial bladder tumors: a prospective, randomized trial. J. Urol. 138: 295.

21. Lamm DL, Crawford ED, Blumenstein J et.al. (1993): SWOG 8795: A randomized comparison of Bacillus Calmette-Guérin and Mitomycin-C prophylaxis in stage Ta and T1 transitional cell carcinoma of the bladder. J.Urol. 149:4 (275).

EFFECTS OF ISONIAZID ON THE BCG-INDUCED LOCAL IMMUNE RESPONSE AFTER INTRAVESICAL BCG-THERAPY FOR SUPERFICIAL BLADDER CANCER

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ABSTRACT

Because recent investigations showed that the use of Isoniazid severely impaired the local immune reaction to intravesical bacillus Calmette-Guérin (BCG) in the bladder of guinea pigs, in this study the effect of Isoniazid in man has been investigated. Patients were treated with BCG with or without oral Isoniazid. The concentration of free Isoniazid in most urine samples of patients treated with BCG/Isoniazid was much higher (mean 38.0±60.9 μg Isoniazid/ml) than the minimal inhibitory concentration (MIC; 0.1 μg Isoniazid/ml), suggesting at least a bacteriostatic potential of the Isoniazid present. However, *in vitro* studies showed that these urinary concentrations of Isoniazid did not kill BCG organisms effectively, even at a concentration of 150 μg/ml for 24 hours. After the fifth and sixth BCG-instillations a significant increase in the concentration of cytokines (IL2, IL6, IL8 and TNFα), IgG and IgA antibodies to BCG and the number of leukocytes in urine was observed. The leukocytes mainly consisted of granulocytes, besides monocytes/macro-phages and, in lower amounts, T- and B-lymphocytes and natural killer (NK) cells. The absolute number of granulocytes and the concentration of IgG antibodies after BCG-instillation were significantly suppressed by Isoniazid, whereas Isoniazid appeared to have no effect on the urinary cytokine and IgA antibody concentrations or the total number and phenotype of the leukocytes present. In conclusion, the results of this study indicate that Isoniazid does not impair the local immunological stimulation after BCG-instillation in man as severely as was observed in the guinea pig and it may be expected that Isoniazid does not impair the antitumor efficacy of BCG.

Intravesical bacillus Calmette-Guérin (BCG) therapy is one of the most effective therapies for both prophylaxis and treatment of superficial bladder cancer.[17] BCG belongs to the mycobacteria and is an attenuated strain of the bovine tubercle bacillus. The mode of action of BCG is probably based on local stimulation of the immune system,[11,23] but the actual mechanisms by which BCG mediates antitumor activity are not clearly understood.

Although BCG has lost its virulence, complications of intravesical BCG have been reported.[16 28] Side effects after intravesical instillation of BCG can be local (cystitis) or systemic, such as granulomatous prostatitis hepatitis, pneumonitis and even lethal sepsis.[15] To diminish the local irritating bladder symptoms and to prevent systemic side effects, some urologists prescribe the bacteriostatic drug Isoniazid prophylactically. It has been assumed that Isoniazid will not influence the antitumor effect in man. However, the bacteriostatic potential of Isoniazid may reduce the effective dose of viable BCG organisms and thus reduce the antitumor activity.[13] Recently the effect of Isoniazid treatment on the immune response after repeated intravesical BCG-instillation has been studied in guinea pigs.[5] In that study the administration of Isoniazid severely impaired the immunological stimulatory effects of BCG. The induction of a mononuclear cell infiltrate in the bladder wall was reduced. Enlargement of the regional lymph nodes, and an increase of MHC Class II expression on the lymph node cells, normally observed after intravesical BCG-administration, were inhibited by Isoniazid. Systemic immunity to mycobacteria was also diminished.

The influence of Isoniazid on the antitumor efficacy is now investigated in man (European Organisation for Research and Treatment of Cancer protocol 30911: comparative study of intravesical instillation of epirubicin, BCG or BCG plus Isoniazid in intermediate and high risk pTa-pT1 papillary carcinoma of the urinary bladder).

In previous studies we have investigated the local immunological effects after treatment of superficial bladder cancer patients with BCG by examining the urine on the presence of leukocytes and cytokines.[2,3 4]

In the present study we have investigated the effect of Isoniazid on the local immune response induced by intravesical BCG-administration in patients with superficial bladder carcinoma. In the urine of patients treated with BCG or BCG/Isoniazid the number of cells, the leukocyte subpopulations, and amounts of IL2, IL4, IL6, IL8, TNFα and of class IgG and IgA antibodies

to BCG were compared. The concentration of Isoniazid in the urine and the effect of these concentrations on the viability of BCG were also determined.

MATERIAL AND METHODS

Patient treatment

Urine specimens were obtained from 22 patients with superficial bladder carcinoma (stage pTa, pT1 and/or carcinoma in situ) who were treated with BCG (Tice and RIVM) after transurethral resection of papillary tumor(s). BCG, approximately $5x10^8$ culturable particles, was administered in 50 ml 0.9% saline once a week for six consecutive weeks. Twelve of the 22 patients investigated, also received 300 mg Isoniazid orally the day before the BCG-instillation, 2 hours before instillation and on the day after instillation (instillation 1-6). After treatment with 6 instillations of BCG, patients were followed up by cytoscopy every 3 months.

Collection and preparation of cells from urine

Because previous investigations showed maximal urinary concentrations of cells and cytokines after repeated (>4) instillations,[2,3,4] in this study urine specimens were examined during the 5th and 6th BCG-instillation. Samples were collected before instillation, 2 to 6 hours (pooled specimens) and 24 hours thereafter. The specimens were centrifuged (5 min, 300*g*) and the supernatants were immediately frozen to -20°C and stored at -70°C. Supernatants were afterwards thawed and used for measuring cytokines, antibodies and Isoniazid.

The cellular sediment was suspended in RPMI 1640 tissue culture medium (Gibco Europe B.V., Breda, The Netherlands), supplemented with 10% fetal calf serum (FCS; Gibco), penicillin (100 IU/ml) and streptomycin (100 µg/ml), referred to as complete RPMI. To obtain high cell viability all urine specimens were immediately cooled and processed at 4°C or on melting ice. After washing twice in complete RPMI, viable nucleated nonsquamous cells in urinary sediments were counted by trypan blue (0.5%) exclusion. The cells were used for immunofluorescence staining.

Immunofluorescence staining

Cells ($5x10^5$) were labelled with 100 µl mAb in Dulbecco's Phosphate Buffered Saline (Gibco) with 0.01% NaN_3 and 2% FCS, referred to as $DPBS^+$, in 96-well Microtest III Assay Plates (Becton Dickinson, Etten-Leur, The

Netherlands). Monoclonal antibody (MAb) FK32 (anti-CD15; granulocytes) was kindly provided by Dr. F. Koning (University of Leiden, Leiden, The Netherlands). Other mAbs were obtained from Becton Dickinson: anti-HLE1 (anti-CD45, leukocytes), anti-LeuM3 (anti-CD14, monocytes/macrophages), anti-Leu12 (anti-CD19, B-lymphocytes), anti-Leu4 (anti-CD3, T-lymphocytes), anti-Leu3 (anti-CD4, helper/inducer T-lymphocytes), anti-Leu2 (anti-CD8, suppressor/cytotoxic T-lymphocytes), anti-Leu11c (anti-CD16, NK cells), anti-Leu19 (anti-CD56, NK cells), anti-IL2R (anti-CD25, IL2-receptor), anti-HLA-DR (Ia non-polymorphic), anti-TCR-1 α/β (T-cell receptor-α/β), anti-TCR-γ/δ-1 (T-cell receptor-γ/δ). Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments from rabbit anti-(mouse Ig) (Organon Teknika, West Chester, Pa, USA) were used as second Ab to detect binding of FK32. All other mAbs were conjugated to either FITC or phycoerythrin (PE). As negative controls, mAb of irrelevant specificity (anti-KLH) but corresponding isotypes (Becton Dickinson) or FITC-conjugated rabbit anti-(mouse Ig) mAb only, were used. Labelled cells were finally fixed in 200 µl paraformaldehyde solution (0.25% in $DPBS^+$).

Flow cytofluorometry and analysis

Samples of $1x10^4$ cells were measured with a fluorescence-activated cell sorter (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, California, USA). During measurement the forward scatter threshold was set on channel 52 for background exclusion. Quantification of granulocytes, monocytes/macrophages, and T- and B-lymphocytes was based on cells with forward scatter >200. The fluorescence of the granulocytes and the monocytes/macrophages was determined in the fluorescence histogram of the total cell population. Analysis of lymphocyte subsets was made possible by a selective cell measurement procedure to increase the number of cells within the lymphocyte gate [2] The number of cells within the lymphocyte gate reacting with a specific mAb is expressed as percentage of the $CD45^+$ cells (leukocytes) within the gate. However, in eleven of the fifteen urine sediments of patients treated with BCG and in ten of the twenty sediments of patients treated with BCG/Isoniazid, the leukocytes present just before the BCG-instillation could not be analyzed because of low cell numbers.

Detection of IL2, IL4, IL6, IL8, TNFα and antibodies specific to BCG

IL4, IL6 and TNFα were determined using specific enzyme-linked immunosorbent assays (ELISA) from Medgenix (Fleurus, Belgium).

For detection of IL2 a specific bioassay with the IL2-dependent murine T-cell line CTLL-16 was used as previously described.[12] A human recombinant IL2 preparation was used as a standard (Proleukin; EuroCetus Benelux B.V., Amsterdam, The Netherlands). IL8 was measured with an ELISA according to a procedure described previously.[9] The detection limits of the various assays were 0.1 IU IL2, 2 pg IL4, 3 pg IL6, 5 pg IL8, and 3 pg TNFa per ml urine. The results were standardized to urine creatinine levels (pg/µmol creatinine). Urine creatinine was determined photometrically (500 nm) with a Cobas-Bio centrifugal analysator (Hoffmann-La Roche Ltd., Basle, Switserland), using alkaline pikrate as reagent. The mean creatinine concentration for all samples was 9.76±5.18 (n=252).

Class IgG and IgA antibodies to BCG were determined with an ELISA.[14] For this purpose flat bottomed ELISA microplates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 100 µl carbonate buffer containing 10 µg/ml purified protein derivative (PPD) of *Mycobacterium tuberculosis*.

Isoniazid determination

For determination of Isoniazid, urine was collected just before, 2-6 hours after (pooled specimens), and 24 hours after the BCG-instillation. After centrifugation (5 min, 300g) the supernatant was stored at -70°C until analysis. Analysis of Isoniazid was based on acetylation of Isoniazid with pentafluorobenzoylchloride in an aqueous solution, resulting in the formation of 2-(4-pyridyl)-5-pentafluorophenyl-1, 3, 4-oxadiazole. Quantification was performed using benzoic hydrazide as internal standard. Both derivatives have favourable electron capture properties and can be analyzed with high sensitivity with Negative Ion Chemical Ionization GC/MS, monitoring their molecular ions $[M]^-$.

Effect of urinary Isoniazid concentrations on BCG viability

The direct cidal effect of Isoniazid to BCG was determined in a quantitative suspension test. Approximately 1.2×10^7 colony forming units (cfu)/ml PBS were exposed to 0, 50 or 150 µg/ml Isoniazid. After 24 hours Isoniazid was inactivated with 0.5% pyruvate. Hereafter mycobacteria were cultured on Middlebrook 7H10 medium The number of cfu was counted after 4 weeks of growth at 35°C in 5% CO_2.

Statistical analysis

Significance of differences between the total cell number, number of cells in various cell subsets, cytokine- and antibody-concentrations before and after

treatment within each group of patients (BCG or BCG/Isoniazid) was determined with Two Sample Sign test for Equal Medians.[26] Differences between the percentages of cells (determined by flow cytometric analysis), the number of cells and the amount of cytokines and antibodies in BCG- and BCG/Isoniazid treated patients were statistically determined with Mann-Whitney two sample (non-matched) test.[26]

RESULTS

Urinary Isoniazid concentration and effect on BCG viability

Isoniazid was administered orally the day before, two hours before and the day after BCG-instillation. The concentration of Isoniazid in urine was measured just before, 2-6 hours after (pooled urine specimens), and 24 hours after the fifth BCG-instillation (Figure 1). Although Isoniazid concentrations showed large variations between individual patients, nearly all concentrations were far higher (mean: 38.0±60.9 µg Isoniazid/ml) than the minimal inhibitory concentration (MIC; 0.1 µg/ml) determined for BCG *in vitro* by the agar dilution technique (after 21 days of incubation with Isoniazid, the number of colony forming units of BCG was counted).[14] Only one patient showed no detectable urinary Isoniazid concentrations just before and 24 hours after BCG-instillation. As a negative control, Isoniazid was also measured in urine of two patients treated with BCG only. In these samples no Isoniazid could be detected (data not shown). To study the effect of high Isoniazid concentrations on the viability of BCG, fresh mycobacteria were incubated with 0, 50 and 150 µg/ml Isoniazid, comparable to Isoniazid concentrations found in the urine after the fifth BCG-instillation (Figure 1). Four weeks after addition of Isoniazid during 24 hours to a BCG culture, none of the Isoniazid concentrations tested appeared to be bactericidal to the mycobacteria (Table 1).

Table 1 Effect of Isoniazid on bacillus Calmette-Guerin organisms exposure (24h)

Concentration of Isoniazid (µg/ml)	Number (x 10) of colony-forming units of BCG
0	6.0
50	7.5
150	7.0

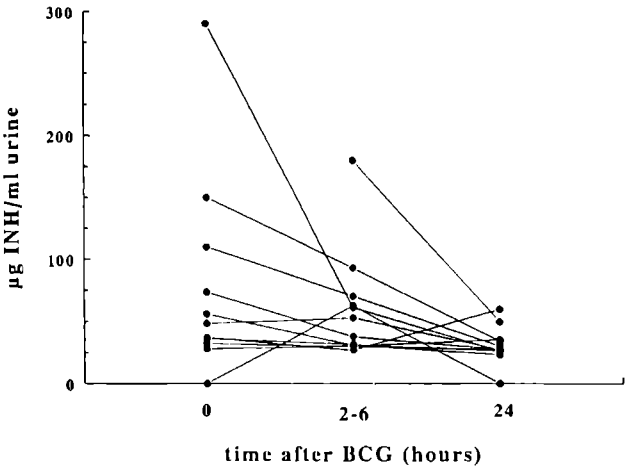

Figure 1. Isoniazid concentration in urine before (0 hrs), 2-6 hrs after and 24 hrs after fifth BCG/Isoniazid instillation.

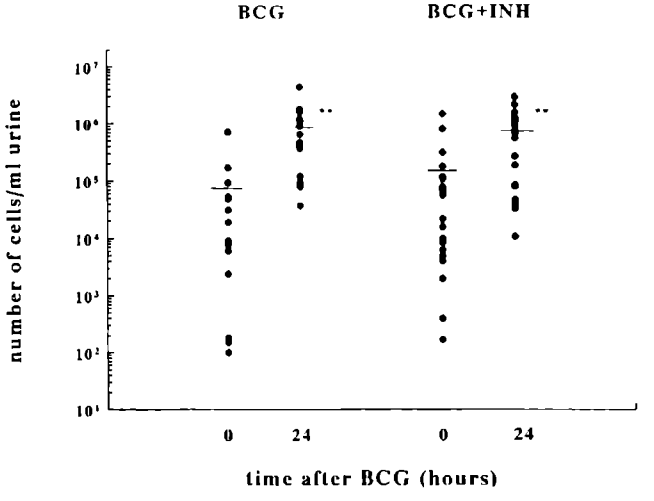

Figure 2. Total number of leukocytes before (0 hrs) and 24 hrs after fifth and sixth BCG and BCG/Isoniazid instillation (determined by trypan blue exclusion). - mean number of cells. The number of cells is significantly increased 24 hours after BCG-instillation, ** $P<0.01$ (two sample sign test).

Effect of Isoniazid on urinary cell numbers

Before the fifth and sixth BCG-instillation, the number of viable cells in urine from patients treated with BCG or BCG/Isoniazid ranged from $1.0x10^2$ to $7.1x10^5$/ml and from $1.7x10^2$ to $1.5x10^6$/ml, respectively. In both groups of patients (BCG and BCG/Isoniazid) the total number of cells in the urine was significantly ($P<0.01$) higher 24 hours after BCG-instillation than before instillation (Figure 2).

Effect of Isoniazid on leukocyte subpopulations

The relative quantities of leukocyte subpopulations of urine sediments before and after the fifth and sixth BCG-instillation, determined by flow cytofluorometry, are presented in Table 2. Granulocytes ($CD15^+$) were predominant (about 67%), in addition to which about 3% of monocytes/macrophages ($CD14^+$), and about 1% T- lymphocytes ($CD3^+$) were found. B-lymphocytes ($CD19^+$) and NK-cells ($CD56^+$) were present in very small amounts or even absent (between 0% and 0.5%).

Table 2 shows that after both BCG and BCG/Isoniazid treatment the percentage of monocytes/macrophages had increased significantly by 24 hours after BCG-instillation compared to pre-instillation values ($P<0.05$). The percentage of T helper/inducer cells ($CD4^+$) was significantly decreased 24 hours after BCG-treatment, but not after BCG/Isoniazid treatment. This decrease was probably caused by high pre-instillation values, in which case it is not relevant. The two groups of patients (BCG and BCG/Isoniazid) did not differ significantly in the percentage of leukocytes either before or 24 hours after BCG-instillation.

For both treatments (BCG and BCG/Isoniazid) the mean percentage of T suppressor/cytotoxic ($CD8^+$) was lower than the percentage $CD4^+$-cells both before and 24 hours after BCG-instillation (Table 2). Before and after BCG or BCG/Isoniazid treatment most of the lymphocytes expressed α/β T-cell receptor (TCR) and a considerable percentage of T-cells showed HLA-DR and IL2-receptor (CD25) expression. No indications were found for different percentages of α/β TCR, HLA-DR or IL2-receptor expression between samples of patients treated with BCG and patients that received BCG/Isoniazid. However, when the absolute number of leukocyte subpopulations are calculated, Isoniazid induced no significant increase of the absolute number of granulocytes 24 hours after BCG-instillation, whereas the absolute numbers of monocytes/macrophages, T-cells and B-cells were significantly ($P<0.05$) increased 24 hours after both treatments (BCG and BCG/Isoniazid) (Figure 3).

Table 2. Leukocyte subpopulations and subsets of lymphocytes in urine before (0 hrs) and after (24 hrs) fifth and sixth BCG-instillation. Percentages given are mean ± s.d. of (n) patients. * P<0.05 (Mann-Whitney) for difference between 0h and 24h.

Cell type	Cell composition (%) after			
	BCG		BCG/Isoniazid	
	0	24h	0	24h
Granulocytes*	68 ± 10 (4)	63 ± 18 (14)	71 ± 13 (10)	65 ± 14 (17)
Monocytes/ macrophages[a]	1 ± 0 (4)	3 ± 2* (14)	2 ± 2 (10)	6 ± 4* (17)
T lymphocytes[b]	1 ± 1 (4)	1 ± 1 (14)	1 ± 1 (10)	1 ± 0 (17)
B lymphocytes[b]	0 ± 0[d] (4)	0 ± 0[d] (14)	0 ± 0[d] (10)	0 ± 0[d] (17)
NK cells[b]	0 ± 0[d] (4)	0 ± 0[d] (14)	0 ± 0[d] (10)	0 ± 0[d] (17)
T helper/ inducer[b,c]	64 ± 13 (3)	38 ± 16* (14)	44 ± 19 (8)	39 ± 15 (17)
T suppressor/ cytotoxic[b,c]	16 ± 2 (3)	18 ± 8 (14)	19 ± 9 (8)	19 ± 11 (17)
IL2 receptor[b,c]	37 ± 32 (3)	16 ± 11 (14)	20 ± 12 (7)	21 ± 17 (17)
HLA-DR[b,c]	67 ± 30 (3)	50 ± 21 (14)	47 ± 22 (8)	55 ± 15 (17)
α/βT cell receptor[b,c]	81 ± 13 (3)	58 ± 26 (14)	56 ± 21 (8)	61 ± 21 (17)
γ/δ T cell receptor[b,c]	17 ± 13 (3)	9 ± 9 (14)	5 ± 4 (8)	7±8 (17)
CD4/CD8 ratio	4.0 ± 1.3 (3)	2.5 ± 1.9 (14)	3.3 ± 3.2 (8)	2.5 ± 1.2 (17)

[a] Determined by flow cytometric analysis of the total cell population with forward scatter > 200.
[b] Determined by flow cytometric analysis of the cells in the lyphocyte gate.
[c] Positive cells expressed as percentage of the CD45+ cells.
[d] Percentages 0 - 0.5%.

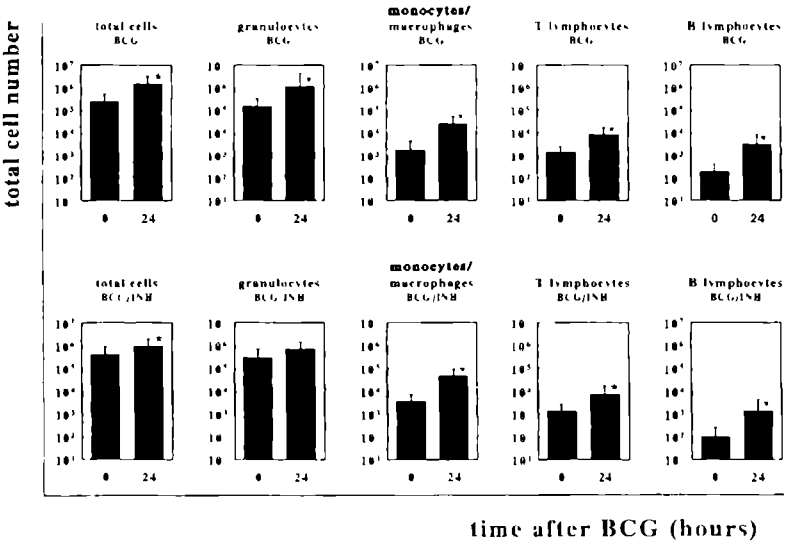

Figure 3. Occurrence of cytokine peak levels in urine during the first 24 hours after BCG-instillation 4-6. Peak level = highest cytokine concentration of the 24 hours after an instillation, evaluated per patient. Frequency of peak levels expressed as percentage of the number of samples of all patients investigated at each time point.

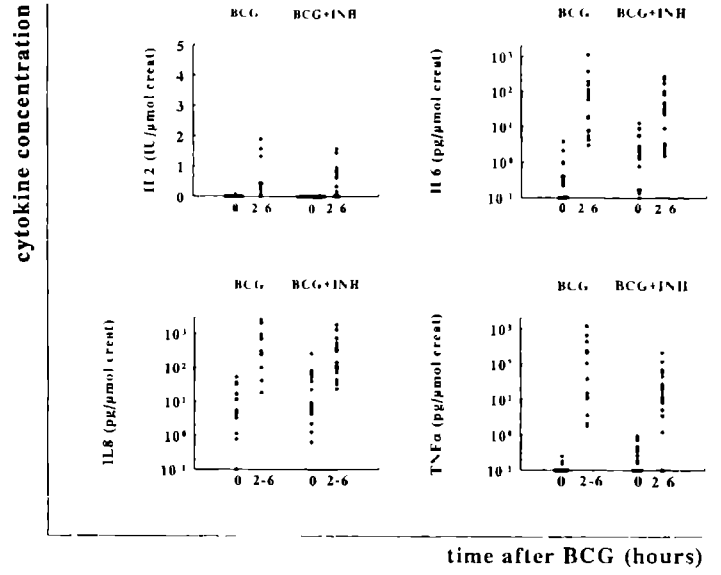

Figure 4. Concentration of cytokines in urine before and after fifth and sixth BCG and BCG/Isoniazid instillation (determined by FI ISA (IL6, Il 8, TNFα) or CTLL16-bioassay (IL2)). Differences between concentrations of these four cytokines 2-6 hrs after and before (0 hrs) instillation are significant ($P<0.01$; Mann Whitney)

Table 3. Increase of concentrations IL2, IL6, IL8 and TNFα after fifth and sixth BCG-instillations, with and without Isoniazid. Values shown are means ± S.D. for *(n)* specimens of concentrations 24h after instillation minus concentration before. Differences are not significant.

Cytokine	Treatment	Increase
IL2	BCG	0.79 ± 1.07 (13)
	BCG/Isoniazid	0.49 ± 0.51 (17)
IL6	BCG	146 ± 283 (15)
	BCG/Isoniazid	61 ± 78 (19)
IL8	BCG	799 ± 883 (15)
	BCG/Isoniazid	345 ± 471 (19)
TNFα	BCG	224 ± 327 (15)
	BCG/Isoniazid	35 ± 53 (19)

Effect of Isoniazid on urinary concentrations of IL2, IL4, IL6, IL8 and TNFα

Figure 4 shows the levels of IL2, IL6, IL8 and TNFα in urine before and 2-6 hours after (obtained from pooled urine samples) the fifth and sixth BCG-instillation. In most pre-instillation samples the concentration of IL2 and TNFα were low or not detectable. In contrast, the levels of IL6 and IL8 in urine were relatively high just before BCG-instillation, ranging from 0 to 12.8 pg IL6/μmol creatinine and 0 to 254 pg IL8/μmol creatinine.

The concentration of urinary IL2, IL6, IL8 and TNFα showed a significant increase during 2-6 hours after BCG-instillation in both groups of patients ($P<0.01$). However, there were considerable variations between individual patients with respect to the concentrations during 2-6 hours after BCG or BCG/Isoniazid treatment. In none of the urine samples tested were detectable IL4 concentrations both before and 2-6 hours after BCG or BCG/Isoniazid found (data not shown). The increase of urinary cytokine concentrations after the fifth and sixth BCG-instillation can be seen from Table 3, showing no significant differences between BCG and BCG/Isoniazid treatment.

Effect of Isoniazid on urinary concentrations of anti-BCG IgG and IgA antibodies

The presence of urinary IgG and IgA antibodies to BCG is presented in Table 4. Anti-BCG IgG and IgA were present before the fifth and sixth instillation of both treatments (BCG and BCG/Isoniazid). The concentration of urinary IgA was significantly increased ($P<0.01$) 2-6 hours after BCG and BCG/Iso-

Table 4. Presence of BCG-specific IgG and IgA antibodies in urine before (0 hrs) and after (2-6 and 24 hrs) the fifth and sixth BCG-instillations [mean ± s.d. for n samples; 1U = [(OD (450 nm)-background OD (450 nm)) / mmol creatinine] x 1000; ** $P<0.01$ (two-sample sign test)]

Treatment	Time h	Concentration (U) of antibodies	
		IgG	IgA
BCG	0	11.20 ± 14.95 (15)	7.63 ± 7.26 (15)
	2-6	87.74 ± 81.27 (15)**	27.82 ± 35.99 (15)**
	24	21.08 ± 25.29 (15)	4.68 ± 5.29 (15)
BCG/Isoniazid	0	28.24 ± 31.32 (20)	6.91 ± 9.35 (20)
	2-6	38.02 ± 41.63 (20)	9.96 ± 11.50 (20)**
	24	10.92 ± 23.38 (20)	2.46 ± 2.90 (20)

niazid treatment. Also IgG was significantly increased ($P<0.01$) 2-6 hours after BCG-treatment, whereas after BCG/Isoniazid treatment no significant increase could be found. By 24 hours after both BCG and BCG/Isoniazid treatment the concentrations of IgG and IgA decreased.

DISCUSSION

In the present study we investigated the effect of oral Isoniazid administration on local cellular immunological stimulation after 5 and 6 intravesical BCG-instillations in man. The great majority of individuals can be characterized as either slow or rapid inactivators of Isoniazid.[19] The large variations observed in Isoniazid concentrations between individual patients may indicate the presence of slow and fast inactivators of Isoniazid in this study (Figure 1). The concentration of free Isoniazid in most urine samples was far higher than the minimal inhibitory concentration as determined for BCG *in vitro* (0.1 μg/ml),[18] suggesting a bacteriostatic potential of the intravesical Isoniazid. The minimal inhibitory concentration was determined by the agar dilution technique, in which the number of cfu of BCG was counted after 21 days of incubation with Isoniazid. As shown in Figure 1, Isoniazid is present in urine only for about 24 hours. The consequences of these temporary high urinary Isoniazid concentrations were studied *in vitro* (Table 1), showing no short term bactericidal activity of Isoniazid to BCG.

Both BCG and BCG/Isoniazid administration induced a significant increase in the total number of leukocytes in the urine 24 hours after BCG-instillation (Figure 2). However, when the cell number after the sixth instillation is determined separately from the fifth instillation, the increase after 24 hours was significantly inhibited by Isoniazid after the sixth instillation, but not after the fifth instillation (data not shown).

The absolute and relative amounts of leukocytes and leukocyte subsets after intravesical BCG therapy are in agreement with observations of De Boer et al..[2] Although the percentage of granulocytes, B-cells and T-cells 24 hours after BCG-instillation showed no significant increase (Table 2), the absolute numbers of the leukocyte subsets were significantly increased after BCG-instillation (Figure 3). These findings are comparable with results reported by De Boer et al..[3] In contrast to the relative low T-cell number in the urine after both treatments (BCG and BCG/Isoniazid), T-lymphocytes are the main cell type present in bladder wall infiltrates in patients after intravesical BCG-administration.[6,20] This is possibly the result of differences in time of sample collection after BCG-treatment. Because T-lymphocytes have been shown to play an important role in the antitumor activity of BCG,[10,21,22] subsets of lymphocytes in urine after intravesical BCG-administration have been characterized. Both before and 24 hours after BCG-instillation, T-cells showed expression of HLA-DR and IL2-receptors, indicating activation of T-lymphocytes. The presence of the activation markers HLA-DR and IL2 receptor is in accordance with their presence in bladder wall biopsies.[6,20] This suggests that the lymphocytes in the urine are probably a reflection of the events which take place in the bladder wall after BCG-administration.

After both BCG and BCG/Isoniazid treatment the levels of IL2, IL6, IL8, and TNFα showed a significant increase during 2-6 hours after BCG-administration. These data are in agreement with results of other studies on cytokines in urine after BCG-treatment.[1,4,7,12,21,24] However, when cytokine concentrations of the fifth and sixth instillation are determined separately, the increase of IL8 and TNFα was significantly reduced by Isoniazid after the fifth instillation (data not shown). After the sixth instillation no significant differences between BCG and BCG/Isoniazid treatment could be observed (data not shown), suggesting probably a transient reduction of the cytokine induction by Isoniazid. The absence of IL4 in urine after both treatments (BCG and BCG/Isoniazid) probably indicates the absence of the Th2 subset of $CD4^+$ T-cells. It has been shown that mycobacteria can selectively induce human T-cells with a Th1-like cytokine secretion profile.[8] The release of BCG-specific antibodies of both IgA and IgG classes after intravesical BCG-

administration was also found by Van Der Sloot et al..[27] Probably, these antibodies may lead to lysis of mycobacteria by opsonization and subsequent increased phagocytosis by granulocytes.[29]

In conclusion, the antitumor activity of BCG has been reported to be dependent on the dose of viable mycobacteria.[13,25] In our study, Isoniazid (tested with concentrations comparable to those found in urine after the fifth instillation) did not kill BCG organisms. Furthermore, Isoniazid did not impair the local immunological effects after BCG-instillation as has been reported in guinea pigs.[5] Although Isoniazid may inhibit the proliferation of BCG because the urinary concentrations found were much higher than the minimal inhibitory concentration, viable BCG organisms are still able to induce the observed local immune response and antitumor activity. If Isoniazid does not influence the immunological action of BCG, it may be expected that Isoniazid does not impair the antitumor efficacy of BCG. If this assumption is true and if Isoniazid would inhibit the potential side effects of BCG, then the use of prophylactical Isoniazid would improve therapy of intravesical BCG in patients with superficial bladder cancer. Both issues are now investigated in man.

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REFERENCES

1. Bettex-Galland M, Studer UE, Walz A, Dewald B, Baggiolini M (1991): Neutrophil-activating peptide-1/interleukin-8 detection in human urine during acute bladder inflammation caused by transurethral resection of superficial bladder cancer and bacillus Calmette-Guérin administration. Eur Urol 19:171

2. Boer EC de, Jong WH de, Meijden APM van der, Steerenberg PA, Witjes JA, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1991): Leukocytes in the urine after intravesical BCG-treatment for superficial bladder cancer: a flow cytofluorometric analysis. Urol Res 19·45.

3. Boer EC de, Jong WH de, Meijden APM van der, Steerenberg PA, Witjes JA, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1991): Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guérin. Cancer Immunol Immunother 33:411.

4. Boer EC de, Jong WH de, Steerenberg PA, Aarden LA, Tetteroo E, Groot ER de, Meijden APM van der, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1992): Induction of urinary IL1, IL2, IL6, and TNF during intravesical immunotherapy with BCG in superficial bladder cancer. Cancer Immunol Immunother 34:306.

5. Boer EC de, Steerenberg PA, Meijden APM van der, Klingeren B van, Jong WH de, Elgersma A, Debruyne FMJ, Ruitenberg EJ (1992): Impaired immune response by Isoniazid treatment during intravesical BCG-administration in the guinea pig. J Urol 148:1577.

6. Bohle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD (1990): Local BCG therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. J Urol 144:53.

7. Bohle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG, Flad HD (1990): Detection of urinary TNF, IL1, and IL2 after local BCG-immunothe rapy for bladder carcinoma. Cytokines 2:175.

8. Haanen JBAG, Waal Malefijt R de, Res PCM, Kraakman EM, Ottenhoff THM, Vries RRP de, Spits H (1991): Selection of a human T helper type 1-like T cell subset by mycobacteria. J Exp Med 174:583.

9. Hack CE, Hart M, Strack Van Schijndel RJM, Eerenberg AJM, Nuijens JH, Thijs LG, Aarden LA (1992): Interleukin-8 in sepsis: relation to shock and inflammatory mediators. Infect and Immun 60:2835.

10. Hanna MGJr, Snodgrass MJ, Zbar B, Rapp HJ (1973): Histopathology of tumor regression after intralesional injection of Mycobacterium bovis:IV Development of immunity to tumor cells and BCG J Natl Cancer Inst 51:1897.

11. Jong WH de, Steerenberg PA, Ruitenberg EJ (1987): Bacillus Calmette-Guérin (BCG) and its use for cancer immunotherapy. Res Monogr Immunol 11:283.

12. Jong WH de, Boer EC de, Meijden APM van der, Vegt PDJ, Steerenberg PA, Debruyne FMJ, Ruitenberg EJ (1990): Presence of interleukin-2 in urine after intravesical treatment of superficial bladder cancer patients with bacillus Calmette-Guérin. Cancer Immunol Immunother 31:182.

13. Kelley DR, Ratliff TL, Catalona WJ, Shapiro A, Lage JM, Bauer WC, Haaff EO, Dressner SM (1985): Intravesical BCG therapy for superficial bladder cancer: effect of BCG viability on treatment results. J Urol Res 143:48.

14. Klein WR, Steerenberg PA, Poelma F, Wiel E van de, Rutten VPM, Misdorp W, Jong WH de, Ruitenberg EJ (1986): Immune reactivity in cattle with ocular squamous cell carcinoma after intralesional BCG-immunotherapy. Cancer Immunol Immunother 22:87.

15. Lamm DL (1992): Complications of bacillus Calmette-Guerin immunotherapy. Urol Clin North Am 19:565.

16. Lamm DL, Meijden APM van der, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A, Debruyne FM (1992): Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial bladder cancer. J Urol 147:596.

17. Meijden APM van der (1991): Non specific immunotherapy with BCG in superficial bladder cancer: an overview. In Vivo 5:599.

18. Meijden APM van der, Klingeren B van, Steerenberg PA, Boer EC de, Jong WH de, Debruyne FMJ (1991): The possible influence of antibiotic therapy on results of Bacillus Calmette-Guérin intravesical therapy for superficial bladder cancer. J Urol 146:444.

19. Peters JH (1960): Studies on the metabolism of Isoniazid. Amer Rev Resp Dis 81:485.

20. Prescott S, James K, Busuttil A, Hargreave TB, Chisholm GD, Smyth JF (1989): HLA-DR expression by high grade superficial bladder cancer treated with BCG. Br J Urol,63:264.

21. Ratliff TL, Haaff EO, Catalona WJ (1986): Detection of interleukin 2 in the urine of patients with superficial bladder tumors after treatment with intravesical BCG. Clin Immunol Immunopathol 40:375.

22. Ratliff TL, Gillen DP, Catalona WJ (1987): Requirement of a thymus dependent immune response for BCG-mediated antitumor activity. J Urol 137:155.

23. Ratliff TL (1992): Role of the immune response in BCG for bladder cancer. Eur Urol 21 (suppl 2):17.

24. Schamhart DHJ, Kurth K (1990): Detection of urinary IL-6 during intravesical BCG therapy (abstract no 531). J Urol 143:321A.

25. Shapiro A, Ratliff TL, Oakley DM, Catalona WJ (1983): Reduction of bladder tumor growth in mice treated with bacillus Calmette-Guérin intravesical and its correlation with bacillus Calmette-Guérin viability and natural killer activity. Cancer Res 43:1611.

26. Siegel S, Castellan NJ (1988): Nonparametric statistics. Mc Graw-Hill, New York.

27. Sloot E van der, Kuster S, Böhle A, Braun J, Wood WG (1992): Towards an understanding of the mode of action of bacillus Calmette-Guérin-therapy in bladder cancer treatment, especially with regard to the role of fibronectin. Eur J Clin Chem Clin Biochem 30:503.

28. Steg A, Adjiman S, Debré B (1992): BCG therapy in superficial bladder tumours - complications and precautions. Eur Urol 21 (suppl 2):35.

29. Teppema JS, Boer EC de, Steerenberg PA, Meijden APM van der (1992): Morphological aspects of the interaction of Bacillus Calmette-Guérin with urothelial bladder cells in vivo and in vitro: relevance for antitumor activity. Urol Res 20:219.

DOES ISONIAZID REDUCE SIDE EFFECTS OF INTRAVESICAL BACILLUS CALMETTE-GUÉRIN THERAPY IN SUPERFICIAL BLADDER CANCER? INTERIM RESULTS OF EUROPEAN ORGANIZATION FOR RESEARCH AND TREATMENT OF CANCER PROTOCOL 30911

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ABSTRACT

Purpose: We analyzed the influence of the tuberculostatic agent Isoniazid on the incidence and severity of adverse effects of intravesical bacillus Calmette Guerin (BCG) therapy in patients with superficial bladder cancer.

Material and methods: In a prospective randomized multicenter study, the side effects of intravesical instillations with Tice-strain BCG with and without Isoniazid were compared in patients with stages pTa and pT1 bladder tumors. Isoniazid was given orally in a dose of 300 mg daily at every instillation in an attempt to decrease the side effects of BCG.

Results: No differences in local or systemic adverse reactions after intravesical immune therapy with BCG could be observed between patients treated with or without prophylactic Isoniazid therapy. However, analysis of liver function tests after BCG with Isoniazid showed slightly more liver toxicity compared to BCG alone.

Conclusions: Prophylactic administration of Isoniazid during BCG-instillations provides no decraese in any known side effect of BCG. In contrast, transient liver function disturbances are encountered slightly more frequently when Isoniazid is administered. The use of prophylactic Isoniazid in patients treated with BCG is not recommended.

Intravesical nonspecific immunotherapy with bacillus Calmette-Guérin (BCG) was introduced in 1976 by Morales et al.,[1] and has proved to be a highly effective treatment of and prophylaxis for recurrent stages pTa and pT1 bladder tumors. Most reports have claimed that BCG is superior to intravesical chemotherapy and, therefore, that it is one of the most effective agents currently available for the prophylactic treatment of stages pTa and pT1 bladder cancer.[2,3] However, in several studies this superiority of BCG intravesical treatment could not be demonstrated.[4,5]

Although BCG has been proved to be very effective, particularly for high risk superficial bladder cancer patients, some urologists are reluctant to use BCG because of the more pronounced adverse reactions reported in the literature.[5] This fear seems to be justified because approximately 5% of patients treated with BCG will have substantial adverse reactions,[6] while 30 to 50% may have minor local or systemic side effects. Therefore, the question remains whether it would be possible to decrease the toxicity of BCG without impairing the antitumorigenic effect. Reduction of the dose of BCG may be possible. Pagano et al. reported that low dose (75 mg) Pasteur strain BCG was as effective as standard dose (150 mg) BCG, but that the toxicity appears to be significantly less.[7] Others have confirmed these results.[8,9] Another possibility could be the prophylactic administration of tuberculostatic agents at each BCG-instillation.

BCG is the attenuated strain of mycobacterium bovis. Although the virulence has been controlled, its antigenetic properties and sensitivity to tuberculostatic agents have remained unimpaired.[10] Tuberculostatic agents, such as Isoniazid, are used to diminish or to treat the local irritative symptoms and systemic side effects caused by intravesical BCG.[11] In 4 different BCG-strains (BCG-RIVM, BCG-Tice, immune Pasteur and Connaught) the susceptibility to Isoniazid and other tuberculostatic agents has been tested in vitro. All BCG preparations were equally susceptible to Isoniazid.[12] Isoniazid is secreted in the urine, which means that if the concentration of Isoniazid in the bladder is appropriate, the drug might be able to kill living BCG bacilli in the bladder. However, the sensitivity of BCG for Isoniazid may influence the antitumorigenic efficacy of BCG-therapy. In animal models the use of Isoniazid has been shown to impair the immunological response of intravesical BCG.[13] A recent study in man indicated that Isoniazid might not impair local immunological stimulation after intravesical BCG-administration.[14] Whether Isoniazid decreases the antitumor effect of BCG in man is unknown.

The precise role of Isoniazid when used prophylactically in patients treated with BCG was subject to investigation in European Organization for Research and Treatment of Cancer protocol 30911. The 2 objectives of this trial were to assess whether Isoniazid decreases local and systemic side effects, as well as the antitumorigenic effect in patients treated with intravesical BCG. We addressed the former question, while the latter cannot yet be answered because the study is still ongoing.

MATERIALS AND METHODS

After complete transurethral resection patients with intermediate and high risk superficial bladder cancer were entered into this 3 arm multicenter randomized phase III study and randomized to receive epirubicin, or Tice-strain BCG alone or with prophylactic Isoniazid. No placebo was used in the BCG only arm so that the study was not blinded with respect to the assessment of adverse events.

Tice-strain BCG ($5x10^8$ bacilli) was administered for 6 consecutive weeks followed by 3 weekly instillations at months 3, 6, 12, 18, 24, 30 and 36, according to the maintenance schedule reported by Lamm et al..[3] The first instillation was administered 7 to 15 days after transurethral resection of the bladder tumors. Isoniazid (300 mg) was given orally on the day before, 2 hours before and the day after each instillation.

The adverse reactions in the BCG arms were divided into local (bladder related) and systemic side effects. The severity of these side effects were classified as none, not requiring a delay, requiring a delay, or requiring cessation of instillation therapy. The complaints and symptoms were recorded before and after each instillation. Specific questions were asked by the urologist or study personnel about the presence or absence of local and systemic side effects. The baseline incidence of local complaints was noted to obtain more insight into the possible adverse effects of BCG-therapy.

Local toxicity was classified as bacterial cystitis – the occurrence of culture proven (not BCG) bacterial cystitis, drug induced or chemical cystitis – irritative symptoms with negative urine culture and other local side effects – hematuria, granulomatous prostatitis, epididymo-orchitis and ureteral obstruction.

Systemic side effects were classified as fever (more than 39^0 C), influenza-like symptoms (general malaise and chills), pneumonitis, hepatitis, cytopenia and sepsis. Skin rash, arthralgia and migratory arthritis were classified as possible allergic reactions.

For evaluation of toxicity patient complaints were noted, urine analysis with urine cultures were performed and liver function tests (alkaline phosphatase, aspartate and alanine aminotransferases, and gamma-glutamyl transpeptidase) were done during each bladder instillation. Based on the severity of adverse effects, the treating physician decided whether no action was to be taken, instillations were to be postponed or BCG-therapy was to be definitively stopped.

From the start of the study in January 1992 until September 1996 (cut off point), a total of 868 patients were entered into this still ongoing study: 289 were randomized to receive Epirubicine, 290 patients and 289 to receive BCG with and without Isoniazid respectively. The toxicity data for BCG alone and with Isoniazid were evaluated. Data concerning the toxicity of epirubicin were not addressed in this analysis.

Toxicity data were available for 436 patients: 224 in the BCG only group and 212 in the BCG plus Isoniazid group. Because the trial is still ongoing and recruiting patients, no followup data were available for recently entered patients. Values which are missing (unknown) caused a slight variation in the total column (number of patients) in the tables. The numbers of treatment cycles (6 or 3 weeks BCG) evaluated in this analysis was 751 for BCG alone and 671 for BCG with Isoniazid.

In each case the maximum degree of toxicity during treatment was assessed. The percentage of patients with a given toxicity in the 2 study arms was compared using a 2 sided chi-square test. For ordered categorical variables, a chi-square test for trend to compare the average maximum degree of toxicity was also done. The maximum change in the liver function tests with respect to baseline was compared using the Kruskal-Wallis test

RESULTS

Local side effects (bladder related)

The local side effects are shown in Table 1. Frequent micturition (more than 1 time per 2 hours) was the most frequent local toxicity observed, and occurred in 119 of 223 (53%) and 105 of 201 (50%) patients given BCG alone or with Isoniazid, respectively (p=0.51). The second most frequent local side effect was chemical cystitis, which occurred in 89 of 223 (40%) and 77 of 221 (36%) patients, respectively (p=0.53). The administration of BCG had to be stopped because of chemical cystitis in 11 patients in each treatment group Bacterial cystitis (culture proved) was observed in 50 of 220 (23%)

Table 1. Local and systemic side effects of intravesical BCG alone versus BCG with Isoniazid.

frequency	< 1 per 2 hours	> 1 per 2 hours	total
BCG	104	119 (53%)	223
BCG plus Isoniazid	106	105 (50%)	211
p = 0 51	210	224 (52%)	434

	No side effects	Side effects: no delay of instillations	Side effects: delay instillations	Side effects stop instillations	total
Chemical Cystitis					
BCG	134 (60%)	70	8	11	223
BCG plus Isoniazid	134 (64%)	53	13	11	211
p=0.53 (%) p=0 97 (trend)	268 (62%)	123	21	22	434
Bacterial Cystitis					
BCG	170 (77%)	26	22	2	220
BCG plus Isoniazid	166 (79%)	22	20	2	210
p=0.74(%) p=0 74 (trend)	336 (78%)	48	42	4	430
Granulomatous prostatitis, epididymo-orchitis or ureteral obstruction					
BCG	163 (73%)	45	9	6	223
BCG plus Isoniazid	152 (72%)	38	11	10	211
p=0 89(%) p=0 38 (trend)	315 (73%)	83	20	16	434

Hematuria	No	Yes	total
BCG	148	75 (34%)	223
BCG plus Isoniazid	151	60 (28%)	211
p = 0 29	299	135 (31%)	434

and 44 of 210 (21%) patients given BCG alone or with Isoniazid, respectively (p=0.74). The instillations were delayed because of bacterial cystitis in 22 and 20 patients, respectively, and had to be stopped in two patients in each group. The incidence of hematuria was also not significantly different between the 2 groups, and occurred in 75 of 223 (34%) and 60 of 211 (28%) patients, respectively (p=0.29). Other known BCG related local side effects, such as granulomatous prostatitis, epididymo-orchitis and ureteral obstruction, had the same incidence in both groups (27% and 28%, respectively, p= 0.89). Thus, the incidence of local side effects did not differ significantly between patients treated with BCG alone or with Isoniazid.

Systemic side effects

Fever of more than 39^0C occurred in 25 of 224 (11%) and 31 of 211 (15%) patients given BCG alone or with Isoniazid, respectively (p=0.34) (Table 1). Influenza-like symptoms (general malaise and chills) were reported in 39 of 224 (17%) and 45 of 210 (21%) patients, respectively (p=0.35).

In regard to allergic reactions, skin rash occurred in 5 of 224 (2%) and 6 of 211 (3%) patients, respectively. Arthralgia and migratory arthritis were not reported in this study. These data show that there is no statistically significant difference in the incidence of the systemic side effects such as fever (greater than 39^0C), influenza-like symptoms and allergic reactions.

However, liver function values for aspartate aminotransferase after treatment were different between the 2 groups. In the group treated with BCG plus Isoniazid, values greater than 1.25 times normal for aspartate and alanine aminotransferases were observed twice as often as in patients who did not receive Isoniazid (Table 2). For aspartate aminotransferase the difference was statistically significant (p=0.05). If only patients with an initial value of aspartate aminotransferase of less than 1.25 times normal are analysed, then 12 of 168 (7%) on BCG alone and 23 of 157 (15%) on BCG plus Isoniazid had an increase in their aspartate aminotransferase values to more than 1.25 times normal during followup (p = 0.05). Patients on BCG plus Isoniazid also showed a greater degree of change in aspartate aminotransferase between the baseline value at entry and the maximum degree of elevation during treatment (p = 0.03). There were no cases of pneumonitis or sepsis.

Table 2. Liver function tests before and during intravesical treatment with BCG alone or with Isoniazid.

	No. Pts. With Value More Than 1.25 Times Normal*/Total (%)		p Value
	BCG Alone	BCG with Isoniazid	
Alkaline phosphatase:			0.81
Before treatment	2/218 (1)	3/216 (1)	
During treatment	15/203 (7)	12/191 (6)	
Aspartate aminotransaminase:			0.05
Before treatment	3/214 (1)	8/215 (4)	
During treatment	14/193 (7)	26/187 (14)	
Alanine aminotransaminase:			0.12
Before treatment	0/217 (0)	3/215 (1)	
During treatment	9/190 (5)	17/181 (9)	
Gamma-glutamyl transpeptidase:			0.81
Before treatment	8/183 (4)	13/185 (7)	
During treatment	28/177 (16)	30/173 (17)	

* For local laboratory.

DISCUSSION

BCG has no direct toxic effect on tumor cells but stimulates a cascade of immune reactions that results in tumor destruction. BCG is a living organism that can produce not only local bladder related and local inflammatory reactions, but also systemic side effects.[6]

Isoniazid is used as a tuberculostatic agents in patients with active lung tuberculosis. One of the objectives of our study was to investigate whether prophylactic short-term Isoniazid administration can decrease the side effects of intravesical BCG-therapy while maintaining antitumorigenic efficacy. In this analysis the side effects of BCG only were compared to those of BCG plus prophylactic Isoniazid administration during 3 days at each instillation.

However, the administration of Isoniazid itself can produce adverse reactions as well, particularly liver function disturbances. The reported adverse reactions after Isoniazid administration used for the treatment of active tuberculosis may include gastro-intestinal complications, peripheral neuropathy and mild neurologic disorders. Liver function enzymes may increase in 10 to 20% of the patients. In most cases this condition is asymptomatic.[15] However, in treating active tuberculosis Isoniazid is administered in a daily dose of 300 mg for a period of 6 to 12 months, often combined with 2 other tuberculostatic drugs to prevent drug resistance to the mycobacteriae.

In our study Isoniazid was given in a dose of 300 mg daily, but only for 3 days during a maximum of 6 consecutive weeks. Although Isoniazid was administered during a maximum of only 18 days the analysis of the liver function tests after its administration with BCG suggested increased liver enzymes compared to the BCG only group. Our results indicate that even short-term administration of Isoniazid may impair liver function. These disturbances, however, are transient (data not shown).

No significant differences in the percentage of patients with adverse events were found for the other side effects analyzed. Based on our 436 patients the study has a power of approximately 90% to detect a difference of 15% and a power of approximately 99% to detect a difference of 20% in the percent of patients with a given side effect.

Some of our results may be influenced by the absence of a placebo in the patients treated by BCG alone, since knowledge of the treatment could have influenced the evaluation and recording of the more subjective side effects in the 2 treatment arms. On the other hand, the measurement of objective parameters, such as the liver function tests, has not been influenced by the trial design. Measurement of side effects remains a subjective issue because symptoms will be experienced differently by individual patients. Moreover, their importance will be assessed differently by different urologists. Some investigators will cease BCG-therapy in certain situations, while others will stimulate their patients to continue treatment because BCG is apparently working. The exact toxicity of BCG-therapy still remains to be defined.[6]

CONCLUSIONS

Our analysis predominantly reflects the immediate toxicity of prophylactic Isoniazid treatment. Most patients were treated with the 6-week induction course followed by 1 or more maintenance courses of three weeks. However,

the majority of patients have not yet reached the second or third year of maintenance therapy. Therefore, it is not yet possible to report the incidence of side effects of prophylactic Isoniazid during the full extent of 3 years of maintenance therapy. However, with regard to the immediate toxicity, use of Isoniazid as a prophylactic agent to diminish the adverse effects of intravesical BCG does not seem advisable.

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1. Morales A, Eidinge D, Bruce AW (1976): Intravavitary bacillus Calmette-Guérin in the treatment of superficial bladder tumors. J.Urol. 116:180.

2. Martinez-Pineiro JA, Leon JJ, Martinez Pineiro L, jr., Fiter L, Mosteiro JA, Navarro J, Garcia Matres MJ, Carcamo P (1990): Bacillus Calmette-Guérin versus Doxorubicin versus Thiotepa: a randomized prospective study in 202 patients with superficial bladder cancer. J.Urol. 143:502.

3. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, Stanisic TH, Smith JA jr., Sullivan J, Sarosdy MF, Crissman JA and Coltman CA (1991): A randomized trial of intravesical doxorubicin and immunotherapy with bacillus Calmette-Guérin for transitional-cell carcinoma of the bladder. N. Engl. J. Med., 325: 1205.

4. Debruyne FMJ, Meijden APM van der, Geboers ADH, Franssen MPH, Leeuwen MJW van, Steerenberg PA, Jong WH de and Ruitenberg JJ (1988): BCG (RIVM) versus Mitomycin intravesical therapy in superficial bladder cancer. First results of randomized prospective trial. Suppl. Urology, 31:20.

5. Witjes JA, Meijden APM van der, Witjes WPJ, Doesburg W, Schaafsma HE, Debruyne FMJ and the members of the Dutch South-East Cooperative Urological Group (1993): A randomized prospective study comparing intravesicalinstillations of Mitomycin-C, BCG-Tice and BCG-RIVM in pTa-pT1 tumours and primary carcinoma in situ of the urinary bladder. Eur.J.Cancer, 29a, 12:1672-1676.

6. Lamm DL, Meijden APM van der, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A, Debruyne FMJ (1992): Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial badder cancer. J.Urol. 47:596-600.

7. Pagano F, Bassi P, Milani C, Meneghini A, Maruzzi D, Garbeglio A (1991): A low dose bacilus Calmette-Guérin regimen in superficial bladder cancer therapy: is it effective? J. Urol. 146:32-35.

8. Blumenstein BA, Lamm DL, Jewett MA, Humphries G, Boux L, Montie JE, Scardino P, Grossman HB, Crawford ED, Smith JA, Stanisic T (1989): Effect of colony-forming unit dose of Connaught BCG on outcome to immunotherapy in superficial bladder cancer. A SWOG study J.Urol. 143:340A (abstract 608).

9. Martinez-Pineiro JA, Solsona E, Flores N, Isorna S (1995): Improving the safety of BCG-immunotherapy by dose reduction. Eur.Urol. 27 (suppl.1).13-18.

10. Guérin C (1980): The history of BCG. In: Rosenthal SR (ed). BCG vaccine: tuberculosis-cancer Littleton Massachusetts: P.S.G. Publishing Company Inc. p. 37.

11. Lamm DL, Steg A, Boccon-Gibbon L, Morales A, Hanna MG, Pagano F, Althan O, Bosman S, Fisher HAF, Jakse G, Chisholm GD, Meijden APM van der, Debruyne FMJ (1989): Complications of bacillus Calmette-Guérin immunotherapy: review of 2602 patients and comparison of chemotherapy complications. In EORTC Genito-urinary Group Monograph 6: BCGin superficial bladdercancer: 335-355. Alan R.Liss, Inc.

12. Meijden APM van der, Klingerden B van, Steerenberg PA, Boer LC de, Jong WH de, Debruyne FMJ (1991): The possible influence off antibiotics on results of Bacillus Calmette-Guérin intravesical therapy for superficial bladder cancer. J.Urol. 146: 444-446.

13. Boer, LC de, Steerenberg PA, Meijden APM van der, Klingeren B van, Jong WH de, Elgersma A, Debruyne FMJ, Ruitenberg EJ (1992): Impaired immune response by Isoniazid treatment during intravesical BCG-administration in the guinea pig. J.Urol. 148: 1577-1582.

14. Stassar MJJG, Vegt PDJ, Steerenberg PA, Meijden APM van der, Meiring HD, Dessens-Kroon M, Geertzen HGM, Otter W den (1994): Effects of Isoniazid (INH) on the BCG-induced local immune response after intravesical BCG-therapy for superficial bladder cancer. Urol. Res. 22: 177-184.

15. Miller B (1993): Preventive therapy for tuberculosis. Med.Clin.N.Am. vol. 77. 1263-1275.

THE PROGNOSTIC VALUE OF E-CADHERIN AND p53 IMMUNOHISTOCHEMISTRY IN PATIENTS WITH SUPERFICIAL BLADDER CANCER

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INTRODUCTION

Transitional cell carcinoma of the urinary bladder can present in two distinct forms, i.e. as superficial and as invasive lesion. The majority of patients present with the relatively benign superficial tumours (approximately 75%), which are limited to the mucosa (pTa) or the lamina propria (pT1).[1] Carcinoma in situ is a histological process that can also be considered superficial, however these lesions tend to behave more aggressive and are often found in association with high grade superficial tumors.

Clinically, patients with superficial bladder cancer represent a heterogeneous group, some having innocuous papillary tumors that have no significant effect on the life expectancy of the patients whereas in other cases the tumors recur often and in the worst case progress to life threatening malignancies. It is the latter phenomenon that occurs in approximately 10% of the patient population and is clinically most relevant. Early and accurate prediction of tumor progression would enable the urologist to install a more radical therapy at a time when it would be truly curable.

Despite all effort undertaken to this day, the clinical and pathological prognostic factors are not sufficient for individualization of therapy. Clearly, the combination of stage, grade, tumor multiplicity and time to recurrence can be used to estimate the chance for recurrences and to a lesser extent of tumor progression.[2,3] However, the specificity of these parameters is not sufficient.

There is an urgent need for progression markers and now the molecular basis of bladder cancer development is better understood, this can be the basis for the design of molecular prognostic factor algorithms. In this respect E-cadherin and p53 immunohistochemistry are considered good candidates.[4,5]

So far, however, only retrospective studies indicated that p53 and E-cadherin immunohistochemistry can provide additional prognostic information with respect to the progression of superficial bladder tumors. We have prospectively followed a group of patients with superficial bladder tumors by cystoscopy and quantitative cytology in the period 1990-1996.[6,7] Now a reasonable number of progression events has occurred we were able to test the prognostic value of E-cadherin and p53 immunohistochemistry on basis of this prospectively recruited cohort of patients. The non-progressive patients were taken from the same cohort.

MATERIAL AND METHODS

Tumor specimens

The tumor specimens analyzed came from patients that were entered in a prospective study designed to assess the clinical utility of a quantitative cytological procedure termed Quanticyt™. In summary, patients with either a newly diagnosed superficial bladder tumor or with a history of superficial bladder cancer were entered in this study and followed by cystoscopy and quantitative cytology in the period 1990-1996.[7]

Until now progression from pTa/pT1/carcinoma in situ to muscle invasive bladder tumors (≥pT2) was documented for 23 patients (19 men, 4 women) All patients received optimal intravesical therapy. Patients were compared to a control group of 22 patients (20 men, 2 women) with superficial tumors, without progression. Tumor specimen from the group of progressive tumors were obtained from the pathology laboratories of the University Hospital of Nijmegen, The Netherlands (13 patients), the Canisius Wilhelmina Hospital Nijmegen, The Netherlands (5 patients) and the Rijnland Hospital Leiderdorp, The Netherlands (5 patients). All tumor specimen from the control patient group were derived from the pathology laboratory of the Rijnland Hospital Leiderdorp. These patients were followed for a period of 63 till 128 months (mean of 102 months) clinically with control cystoscopies. From these patients, 2 tumors resected at different periods per patient were analyzed. The period of time between the two tissue samples taken, varied from 12 until 96 months (mean, 58 months). The time of diagnosis of all tumor tissues was from August 1985 - December 1995.

Immediately after surgical resection, tumor specimens were fixed in a 10% neutral-buffered formalin solution, overnight. Following fixation, 4-μm paraffin tissue sections were stained with hematoxylin and eosin. All tumors were histologically graded according to the WHO criteria by three-tiered system, identifying well, moderately and poorly differentiated tumors. Staging of tumor specimens was according to the TNM-classification. One paraffin block from each period, representative for tumor grade and stage was selected for further immunohistochemical evaluation.

E-cadherin and p53 immunohistochemistry

To enhance E-cadherin and p53 immunohistochemistry in formalin-fixed, paraffin-embedded bladder tumor tissues, sections were treated with microwave antigen retrieval. Briefly, after deparaffinization, sections were blocked for endogenous peroxidase activity by incubation in 3% hydrogen peroxidase

for 30 minutes at room temperature. Slides were rinsed in phosphate-buffered saline after each step. Sections were then submerged in 0.1M citrate buffer, pH 6.0, and heated in a 800 Watt microwave on full power for 2 x 5 minutes cycles, pausing to ensure that there was no fluid loss due to evaporation. After cooling, the sections were blocked with 1% normal horse serum. For E-cadherin, a monoclonal antibody clone HECD-1 (Takara, Berkeley, USA) at a dilution of 1:50 was applied at 4^0C, overnight.

For p53, a monoclonal antibody clone DO7 (Neomarkers, Fremont, CA) at a dilution of 1:100 was applied at 4^0C, overnight. A negative and positive control was substituted in every batch. A Vectastain Elite kit (Vector laboratories, Burlingame, CA) was used for peroxidase visualization.

Scoring of E-cadherin and p53 staining

The light-microscopical distribution of E-cadherin staining was classified according to its localization in the cell by two independent observers (TEGR, TWA) on two separate occasions. Normal bladder epithelium present in the tumor sections was used as an internal positive control. Tumors were classified as normal if discrete staining was present at cell-cell contacts only. Because the most malignant tumor cell population will ultimately determine the patient prognosis, we defined the presence of an aberrant (apical or cytoplasmic location of E-staining) or negative staining in more than 10% of the malignant cells as abnormal.[8]

The accumulation of p53 was classified after semiquantitative analysis at a 200X magnification in the tumor's p53 hot-spot. Staining in more than 10% of the tumor nuclei was defined abnormal.

Statistical analysis

For statistical analysis, the Chi-square and Fisher Exact tests for the comparison between two sample percentages and in the multi variate discriminate analysis the Wilks method was used. A probability (p) value less than 5% was considered as statistically significant. Where p-values are not mentioned, statistical analysis was regarded not reliable due to the small sample size.

RESULTS

The E-Cadherin and p53 expression in tissue specimens from 23 patients (19 men, 4 women) with bladder tumors who progressed from superficial tumors, pTa, pT1 and/or carcinoma in situ to muscle invasive disease, pT2 or higher were analyzed immunohistochemically for expression of p53 and E-cadherin.

Table 1. TNM-classification at initial diagnosis.

	pTa	pT1	primary carcinoma in situ	concomitant carcinoma in situ	total
progressive tumors	6	15	2	6	23
non-progressive tumors	17	5	0	0	22

Table 2. Tumor grading at initial diagnosis.

	grade 1	grade 2	grade 3	carcinoma in situ	unknown	total
progressive tumors	3	4	12	2	2	23
non-progressive tumors	7	13	2	0	0	22

This group comprised 6 pTa-tumors (26%), 15 pT1-tumors (65%), 2 primary carcinoma in situ (9%). In six tumors (26%), concomitant carcinoma in situ was observed. Histopathological grading showed 3 well differentiated (13%) tumors, 4 were moderately differentiated (17%) and 12 poorly differentiated (52%). The relative pathological stage distribution in the control group was as follows: pTa: 17 tumors (77%); pT1: 5 tumors (23%). These were graded as well differentiated tumors (32%) in 7 cases, as moderately differentiated (59%) in 13 patients and in 2 as poorly differentiated tumors (9%) (Table 1, 2).

In the group of patients with progressive disease, abnormal E-cadherin expression was found in 11 cases, whereas 4 tumors showed normal staining patterns. For technical reasons (fixation damage) in 8 tumors the E-cadherin expression could not be assessed since the internal control was not positive (Table 3). The E-cadherin staining in the control group was more reliable, i.e. assessment of E-cadherin expression could be performed in 91% of all cases that all showed normal E-Cadherin expression (Table 3).

Table 3. E-cadherin expression in progressive and non-progressive superficial bladder tumors.

	normal	abnormal	not evaluable	total
progressive	4	11	8	23
non-progressive	20	0	2	22

The p53 expression could be analyzed in all tumors. In the group of patients with proven progressive disease, thirteen showed an overexpression of the p53 protein, whereas in 10 tumors a normal p53 expression was found (Table 4).

The control group showed overexpression of the p53 protein in 10 tissue samples (45%). In 1 patient, overexpression was seen in the first tumor analyzed only, in 3 patients in the second tissue sample and in 3 patients (3%) the overexpression was determined in both tumors. A normal p53 expression was seen in 10 tumors (45%) and in 2 tumors the p53 status could not be determined since the immunohistochemistry results did not comply with quality control (Table 4).

Statistical analysis of the classical tumor parameters, tumor grade, tumor stage and the presence of carcinoma in situ confirmed their prognostic value concerning the risk of tumor progression. The p-values found, were 0.0028 for tumor grade, 0.0019 for tumor stage and 0.0023 for the presence of carcinoma in situ. Similarly, the presence of abnormal E-cadherin expression in the superficial bladder tumors could be classified as a prognostic parameter concerning the risk of tumor progression (p=0.00001). Hence, E-cadherin immunohistochemistry was the most powerful prognostic parameter. Using the predetermined scoring system we were unable to confirm that overexpression of p53 had prognostic value for tumor progression, (p=0.4573).

Table 4. p53 expression assessed immunohistochemically in progressive and non progressive superficial TCC of the bladder.

	normal	abnormal	not evaluable	total
Initial superficial tumors from progressive cases	10	13	0	23
Non-progressive tumors	10	10	2	22

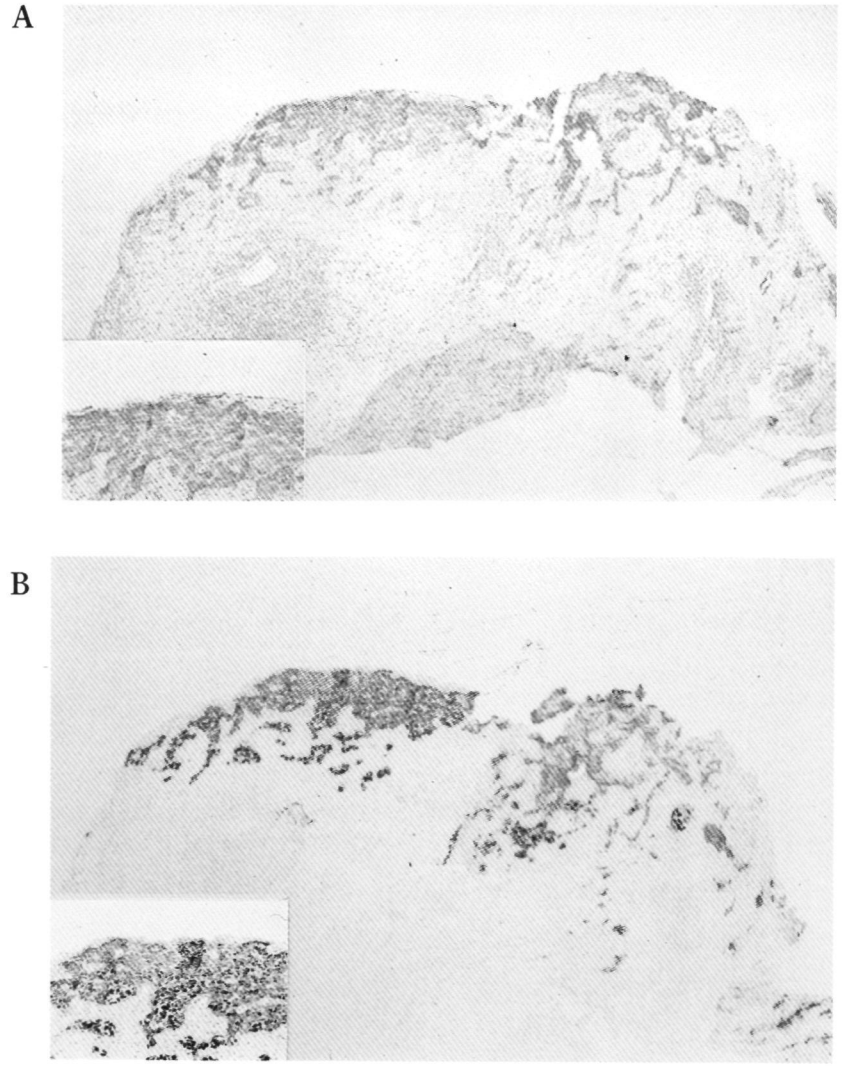

Figure 1. Representative photomicrographs of abnormal E-cadherin and p53 immunohistochemical stainings. Panel A: E-cadherin. Note the diffuse staining NOT localized at cell-cell contacts. Panel B: p53. Note that in the cancer area 50% of the nuclei are stained **positive. Magnification 200 x, inset 1000x.**

In the multivariate discriminate analysis (F-to enter=3, Wilks method) of the entire group, the combination of tumor grade and the presence of carcinoma in situ showed to be of prognostic value. The sensitivity of this combination was 70%, the specificity was 96%. Thus, this combination of parameters shows lower sensitivity and specificity than E-cadherin immunohistochemistry alone.

DISCUSSION

The classical tumor parameters like tumor grade, tumor stage and the presence of carcinoma in situ are the standard prognostic factors used in the treatment of patients with bladder cancer. The clinical value of these parameters has been confirmed in this study. Unfortunately, even the combination of grade and the presence of carcinoma in situ identifies future tumor behavior only in 82% of all patients.

New prognostic markers may be helpful in individualizing the treatment of patients with bladder cancer. The molecular markers E-cadherin and p53 have shown to have prognostic value in patients with bladder cancer.[4,5] In the non-progressive tumor group in none of the 22 tumors studied an abnormal E-cadherin expression could be detected and in the group of 23 superficial tumors which progressed to muscle invasive disease in 15 the E-cadherin status could be assessed reliably. Of these, 11 tumors showed an abnormal E-cadherin expression pattern, which means that the presence of an abnormal E-cadherin expression has significant prognostic value in regard to tumor progression (sensitivity 73%; specificity 100%). These data are in agreement with the data from Bringuier et al.[8] and Otto et al..[9] The main concern is that a significant number of tumors (8/23) were not suitable for reliable E-cadherin scoring, i.e. the normal urothelium was not homogeneously positive. Tissue fixation is likely causing this problem and recently we developed a new formalin fixation protocol optimized for minimal chance for antigen damage by overfixation.

The p53 data are more controversial. Several retrospective studies have shown that p53 immunohistochemistry has prognostic value to predict the progression of superficial bladder cancer.[10,11] The controversy may be explained by the fact that we decided to use a scoring system in which the threshold was based on the expression of p53 in normal urothelium. The score was based on the average number of positive nuclei (scored in ten cases) plus three times the standard deviation (10% of the nuclei positive). When we used another cutoff level similar to that of Sarkis and colleagues,[10 11] i.e. 25% of nuclei positive, there was a clear trend towards a significant prognostic value of p53 immunohistochemistry. This once more indicates that assessment of p53 status by immunohistochemistry is more finicky and standardization of methods and scoring system is urgently needed. This is further corroborated by the data of Vet et al..[12] concerning the prognostic value of p53 mutation analysis in the same group investigated here. In that study, p53 mutations specifically predicted the chance for tumor progression. Moreover, the mu-

tations were found up until 36 months prior to the manifestation of tumor progression.

CONCLUSIONS

In this analysis of patients that were prospectively followed the prognostic value of classical tumor parameters; tumor grade, tumor stage and the presence of carcinoma in situ was confirmed. In addition, abnormal E-cadherin expression showed to be a marker with a high potential to predict tumor progression, albeit that the method needs optimization on paraffin embedded material. Under the conditions we used, i.e. immunohistochemistry and a threshold of 10% of nuclei positive, p53 appeared to have no significant prognostic value. In the treatment of patients with superficial bladder cancer the analysis of the E-cadherin status of the tumor can be helpful in making the decision when a more aggressive treatment modality has to be advised.

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REFERENCES

1. Prout GR, Jr. (1977): Bladder carcinoma and a TNM system of classification. J. Urol. 117: 583-590.

2. Kiemeney LA, Witjes JA, Heijbroek RP, Verbeek AL and Debruyne FMJ (1993): Predictability of recurrent and progressive disease in individual patients with primary superficial bladder cancer. J. Urol. 150: 60-64.

3. Witjes JA, Kiemeney LA, Oosterhof GO and Debruyne FMJ (1992): Prognostic factors in superficial bladder cancer. A review. Eur. Urol. 21: 89-97.

4. Giroldi LA, Bringuier PP, Schalken JA (1994): Defective E-cadherin function in urological cancers: clinical implications and molecular mechanisms. Invasion Metastasis 14: 71-81.

5. Cordon Cardo C and Fleischmann J (1997): Molecular and immunopathology studies of oncogenes and tumor supressor genes in bladder cancer. World J Urol. 15: 112-119. Bibliography.

6. Vet JA, Witjes JA, Marras SE, Hessels D, Poel HG van der, Debruyne FMJ, Schalken JA (1996): Predictive value of p53 mutations analyzed in bladder washings for progression of high-risk superficial bladder cancer. Clin Cancer Res. 2: 1055 1061.

7. Van der Poel HG, Witjes JA, Stratum P van, Boon ME, Debruyne FMJ and Schalken JA (1996): Quanticyt: karyometric analysis of bladder washing for patients with superficial bladder cancer. Urology 48: 357-364.

8. Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FMJ and Schalken JA (1993): Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. Cancer Res. 53: 3241-3245.

9. Otto T, Birchmeier W, Schmidt U, Hinke A, Schipper J, Rubben H and Raz A (1994): Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas. Cancer Res. 54: 3120-3123.

10. Sarkis AS, Dalbagni G, Cordon Cardo C, Zhang ZF, Sheinfeld J, Fair WR, Herr HW and Reuter VE (1993): Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. J. Natl. Cancer Inst. 85: 53-59.

11. Sarkis AS, Dalbagni G, Cordon Cardo C, Melamed J, Zhang ZF, Sheinfeld J, Fair WR, Herr HW and Reuter VE (1994): Association of p53 nuclear overexpression and tumor progression in carcinoma in situ of the bladder. J. Urol. 152: 388-392.

12 Vet JA, Witjes JA, Marras SE, Hessels D, Poel HG van der, Debruyne FM, Schalken JA (1996): Predictive value of p53 mutations analyzed in bladder washings for progression of high-risk superficial bladder cancer. Clin.Cancer Res. 2: 1055-1061.

SUMMARY

SUMMARY

This thesis describes different studies concerning intravesical BCG immunotherapy to try to improve this therapy in patients with superficial bladder cancer.

The rationale of this thesis is given in **chapter 1.**
In **chapter 2,** BCG in superficial bladder cancer, consensus and controversies, items are discussed on which consensus has been reached as well as the problems which have to be solved or on which controversies exist concerning BCG treatment in patients with bladder cancer. The use of BCG in bladder cancer in man was first published by Morales et al. in 1976. This therapy turned out to be very successful. With the introduction of BCG therapy, many questions did rise, like: what is the best route of administration, which BCG strain is the most appropriate one, what is the best treatment schedule with which dose and how can the side effects observed after BCG administration be minimalized.

The most thoroughly investigated route of administration is the intravesical instillation, initially combined with percutaneous administration. At this moment percutaneous supportive inoculation has been abandoned. It is considered as not essential for the antitumor effect of BCG therapy in bladder cancer. In most centres intravesical administration of BCG alone is accepted as the best route of administration in the treatment of patients with superficial transitional cell cancer of the bladder.

In searching for the appropriate BCG strain, so far the results of seven substrains for immunotherapy in superficial bladder cancer have been published. These preparations are Pasteur (France), Armand-Frappier (Canada), Connaught (Canada), Tice (USA), Evans (UK), Moreau (Brazil) and RIVM (The Netherlands). Two procedures for culturing BCG bacteria for bladder cancer treatments are available. The mycobacteria can be grown as a pellicle on the surface of a liquid medium. At harvesting, the pellicle is ground in a ball mill to a paste. The final product of this procedure contains not only living BCG bacteria but also dead microorganisms and subcellular debris. BCG bacteria can also be grown in a homogeneously stirred deep culture system. This culture method results in a relatively high ratio of viable organisms and a small quantity of subcellular debris and dead bacilli. Most of the BCG preparations are surface cultured. Evans and BCG-RIVM strains are produced according to the homogeneous culture method. At this moment too few studies have been performed to reveal the best strain

In finding the optimal treatment schedule, two basically different instillation schedules have been used so far. The induction scheme which consists of six consecutive weekly instillations and the so-called maintenance scheme The maintenance scheme starts with an induction course of also six consecutive weekly instillations followed by repeated instillations during months up to three years. The theory behind these repeated instillations is the assumption that these "booster" instillations evoke a renewed immune response against the tumors. Regarding the results of the reported literature, there is strong evidence that maintenance therapy is superior to induction therapy alone.

To evaluate the optimal dose for BCG therapy in patients with bladder cancer, different studies are ongoing. At this moment no consensus has been reached about the question what the most optimal dose for BCG is. This also is the fact for the question how the toxicity of BCG treatment can be minimalized. Trials in which the prophylaxis of isoniazid is used, have started, see chapter 6 of this thesis.

The **conclusion** of chapter 2 is that although BCG has been proved to be very effective in the treatment of patients with superficial bladder cancer it is certainly not a panacea for all patients with superficial bladder cancer and more research is needed to answer the unsolved problems, like what is the optimal dose of BCG and which BCG strain is superior

Despite the widespread use of BCG, the way in which BCG exerts its antitumor activity is still not well understood. The rationale of the BCG-treatment is to activate the immune system, ending up in an immunological reaction against locally present bladder tumors cells and tumor cell degradation. To try to understand this mechanism a little better, the immunological products observed in the urine of superficial bladder cancer patients after intravesical immune therapy with BCG are studied and described in **chapter 3**. In this chapter it has been shown, that after intravesical BCG administration in patients a local reaction of the immune system occurs. The number of leucocytes in the urine increases markedly. Fluorescence activated cell sorters (FACS) analysis of these leucocytes has revealed that in addition to the predominantly present granulocytes, also monocytes/macrophages and lymphocytes can be detected. The large number of granulocytes, which apparently accumulate in the urine, represent the first line of defence against the mycobacteria. The lymphocytes present are mainly T-lymphocytes. The relative numbers of cells of leukocyte sub-populations are confirmed by light microscopical examination. As the antitumor activity of BCG is assumed to be T-cell-mediated, the subset composition and activation status of the

lymphocytes present in the urine after intravesical BCG therapy are also investigated in this chapter. The T-lymphocytes are mostly of the CD4+ (helper/inducer) phenotype, the remainder being CD8+ (suppressor/cytotoxic) T-cells. NK-cells and B-cells are also present and HLA-DR antigens are present on the lymphocytes, indicating a high activation status of the T-cells after intravesical BCG treatment. The induction of interleukines in the urine: IL1, IL2, IL6 and TNFα during intravesical BCG therapy, have been demonstrated. The presence of IL2 indicates activation of T-cells. IL1, IL6 and TNFα may be produced by BCG-activated monocytes/macrophages, however, production by other cells cannot be excluded. In this studies peak concentrations of IL1, IL2, IL6 and TNFα were most frequently present in urine specimens collected from two to six hours after BCG instillations four to six.

The **conclusions** of chapter 3 are that the clear increase in the number of granulocytes, monocytes/macropges and T-lymphocytes which were observed in the urine 24 hours after BCG instillations, indicate local activation of the immune system. The detection in the collected urine samples of IL2 are considered as the results of activation of BCG-specific T-cells. The presence of IL1, IL6 and TNFα might suggest activation of macrophages.

The combination of the observed leucocytes and cytokines may play an important role in the antitumor activity of BCG against bladder cancer. The presence or absence of cytokines may also have prognostic value concerning the clinical response on BCG therapy.

The analysis of immunological products in the urine of superficial bladder cancer patients after intravesical immunotherapy with bacillus Calmette-Guérin contribute to understand the actual effects of the antitumor activity of BCG. The efficacy of BCG therapy is proven in clinical studies. However, it is still unknown which BCG strain is superior (see chapter 2).

In **chapter 4** the results of a randomized prospective study are reported in which mitomycin-C, BCG-Tice strain and BCG-RIVM strain are compared in patients with primary or recurrent superficial bladder tumors, including carcinoma in situ. The followup varies from 2 till 81 months (mean 36 months). After a complete transurethral resection of all visible tumors the patients are treated with mitomycin-C (30 mg) once a week for 4 consecutive weeks and thereafter every month for a total of 6 months, BCG-Tice or BCG-RIVM (5×10^8 colony forming units) were instilled once a week for 6 consecutive weeks. The treatment efficacy and the incidence of side effects in 437 patients were reported.

The analysis of efficacy in the group of patients with papillary tumors shows a statistically significant difference between the treatment arms (p=0.04). The mitomycin-C and BCG-RIVM treatments were equally effective (p=0.53), but mitomycin-C treatment was more effective than BCG-Tice (p=0.01).

For carcinoma in situ-tumors the complete response was analysed as parameter of efficacy. In all patients with carcinoma in situ-tumors the complete response was 68% (34/50) for a duration of 2 till 49 months (mean 19 months). In the mitomycin-C-group the complete response was 67% (8/12), in the BCG-Tice group 74% (17/23) and in the BCG-RIVM group 60% (9/15). These results are not statistical significant (chisquare test, p=0.66).

The conclusion of this study is that 6 months of intravesical chemotherapy with mitomycin-C in our study is more effective in comparison with 6 weeks immunotherapy using BCG-Tice. Mitomycin-C causes significantly less side effects than both of the BCG strains.

The use of isoniazid impaires the local immune reaction to intravesical BCG in the bladder of guinea pigs. In **chapter 5** the effect of prophylactic given isoniazid in man has been investigated. Patients were treated with BCG with or without oral isoniazid. The concentration of free isoniazid in most urine samples of patients treated with BCG plus isoniazid was much higher (mean 38.0±60.9 µg isoniazid/ml) than the minimal inhibi tory concentration (0.1 µg isoniazid/ml), suggesting at least a bacteriostatic potential of the isoniazid present. However, in vitro studies showed that these urinary concentrations of isoniazid did not kill BCG organisms effectively, even at a concentration of 150 µg/ml for 24 hours. After the fifth and sixth BCG instillations a significant increase in the concentration of cytokines (IL2, IL6, IL8 and TNFα), IgG and IgA antibodies to BCG and the number of leukocytes in urine was observed. The leukocytes mainly consisted of granulocytes, besides monocytes/macrophages and, in lower amounts, T- and B-lymphocytes and NK-cells. The absolute number of granulocytes and the concentration of IgG antibodies after BCG instillation were significantly suppressed by isoniazid, whereas isoniazid appeared to have no effect on the urinary cytokine and IgA antibody concentrations or the total number and phenotype of the leukocytes present.

The **conclusion** from this chapter indicate that isoniazid does not impair the local immunological stimulation after BCG instillation in man as severely as was observed in the guinea pig and it may be expected that isoniazid does not impair the antitumor efficacy of BCG. If this assumption is true and if

isoniazid would inhibit the potential side effects of BCG therapy, than the use of prophylactic isoniazid would improve treatment of intravesical BCG in patients with superficial bladder cancer.

To test this hypothesis, the influence of the isoniazid on the adverse effects of intravesical BCG therapy in patients with superficial bladder cancer is studied and reported in **chapter 6**. The aim of this study is to analyse the influence of the tuberculostatic agent isoniazid on the adverse effects of intravesical BCG therapy. In a prospective randomized multicentre study, intravesical instillations with BCG-Tice and BCG-Tice plus isoniazid therapy were compared in patients with pTa and pT1 bladder tumors. Isoniazid was given orally in a dose of 300 mg daily at every instillation. The purpose of this prophylactic administration was to reduce the side effects of BCG.

No differences in adverse reactions after intravesical immune therapy with BCG could be observed between patients treated with or without prophylactic isoniazid therapy, neither locally nor systemically. However the analysis of the liver function tests after BCG plus isoniazid shows more liver toxicity when compared to BCG administration alone.

The **conclusion** of this study is that prophylactic administration of isoniazid during BCG instillations provides no reduction of any of the known side effects of BCG. In contrast transient liver function disturbances are encountered more frequently when isoniazid is administered. The use of prophylactic isoniazid in patients treated with BCG is not to be recommended.

With a more defined indication for the use of intravesical BCG therapy, the results will improve. The conventional prognostic parameters for patients with superficial bladder cancer cannot predict which tumor will behave aggressive in the individual patient. New tumor related markers for these patients are needed to help the urologist in making his decision for more aggressive therapy. **Chapter 7** describes the value of the conventional prognostic parameters and two new markers, the cell adhesion molecule E-cadherin and the tumor suppressor protein p53 in patients with superficial bladder cancer. The prognostic value of the conventional parameters was confirmed. The presence of an abnormal E-cadherin expression in the bladder tumors showed to have a clear prognostic value concerning tumor progression ($p=0.0001$) and is considered as a powerful new prognostic parameter. The over expression of p53 protein did not show to have prognostic value for tumor progression in this study.

The **conclusion** of chapter 7 is that in the treatment of patients with

superficial bladder cancer the evaluation of E-cadherin expression in the tumor has prognostic value and can be helpful for the urologist by making a decision for more aggressive therapy like BCG intravesical immunotherapy.

SAMENVATTING

Patiënten met oppervlakkige blaastumoren – dat wil zeggen, met tumoren die niet in de blaasspier ingroeien – kunnen op verschillende manieren behandeld worden. Een belangrijke behandelingswijze is de *intravesicale immunotherapie met BCG*. Deze therapie komt erop neer dat geprobeerd wordt het afweersysteem van patiënten te activeren door hun via de blaas een verzwakte vorm van het tuberculosebacil, *het Bacil Calmette-Guérin (BCG)*, toe te dienen.

Dát oppervlakkige blaastumoren met deze therapie effectief bestreden kunnen worden staat vast. Over het hoe en waarom van deze behandelingswijze bestaat echter nog veel onduidelijkheid. Een aantal van de vragen waarmee deze therapie is omgeven zal in dit proefschrift worden beantwoord.

In het inleidende **hoofdstuk 1** worden de onderzoeken beschreven waarop dit proefschrift is gebaseerd.

Voordat de uitkomsten hiervan worden gepresenteerd volgt in **hoofdstuk 2** eerst een overzicht van de stand van kennis. Over welke zaken bestaat in de wetenschappelijke literatuur overeenstemming en welke zijn nog onderhevig aan discussie?

De succesvolle toepassing van de BCG-therapie is voor het eerst beschreven in 1976 door Moralis et al.. De introductie van de therapie riep evenwel veel vragen op: hoe kan BCG het best worden toegediend, in welke dosering, welke BCG-stam is het meest geschikt en hoe kunnen de bijwerkingen van de behandeling worden beperkt? Op deze vragen heeft het onderzoek zich nadien gericht.

Van alle mogelijke toedieningsvormen is de blaasspoeling met BCG (*intravesicale instillatie*) het meest intensief onderzocht. Aanvankelijk werd toediening via de blaas gecombineerd met toediening via de huid (*percutane toediening*). Deze laatste vorm wordt nu niet meer toegepast omdat ze geen essentiële bijdrage bleek te leveren aan de bestrijding van blaaskanker. Toediening van BCG uitsluitend via de blaas wordt thans algemeen beschouwd als de beste aanpak.

Behalve aan de toedieningsvorm, is veel onderzoek gewijd aan de vraag welke BCG-stam het meeste effect sorteert. Tot dusverre zijn zeven BCG-stammen beproefd: de *Pasteur-stam* (Frankrijk), de *Armand Frappier-stam* (Canada), de *Connaught-stam* (Canada), de *Tice-stam* (Verenigde Staten), de *Evans-stam* (Verenigd Koninkrijk), de *Moreau-stam* (Brazilië), alsmede de BCG-stam die in Nederland wordt geproduceerd door het Rijksinstituut voor

Volksgezondheid en Milieuhygiene (RIVM). BCG-stammen kunnen op twee manieren worden geproduceerd. Sommige worden gekweekt als oppervlaktecultuur, waarbij zij groeien als een vlies aan de oppervlakte van een vloeistofmedium. Dit is de meest gangbare methode. Andere stammen, waaronder de Nederlandse, worden vervaardigd als een homogene cultuur. Welke productiewijze de beste BCG-stam oplevert is vooralsnog niet duidelijk.

Ook het behandelingsschema is onderwerp van studie geweest. Tot nu toe zijn er twee verschillende toedieningschema's gebruikt. Enerzijds het *inductieschema*, dat bestaat uit zes wekelijkse blaasspoelingen met BCG en anderzijds het *onderhoudsschema*. Het laatstgenoemde begint eveneens met zes wekelijkse spoelingen, maar deze worden gevolgd door aanvullende instillaties gedurende een periode van enkele maanden tot drie jaar. De ratio van de aanvullende blaasspoelingen is dat deze werken als *booster-instillaties* (instillaties die een versterkende werking hebben op de afweerreacties).Voor zover we nu weten, worden met het onderhoudsschema betere resultaten geboekt dan met het inductieschema.

Wat de dosering betreft, op dit moment zijn nog diverse klinische studies gaande naar de vraag in welke dosering BCG het best kan worden gebruikt Over de manier waarop de toxiciteit (de schadelijke bijwerkingen) van de BCG-behandeling kan worden beperkt valt thans evenmin weinig met zekerheid te zeggen. Klinische studies, waarbij *isoniazide* (een geneesmiddel dat wordt gebruikt bij de behandeling van patienten met actieve tuberculose) uit voorzorg wordt toegediend, zijn inmiddels van start gegaan.

Ook over de vraag *hoe* BCG nu precies werkt, is het laatste woord nog niet gezegd. Men neemt aan dat door toediening van BCG in de blaas het immuunsysteem van de patient zodanig wordt geactiveerd dat er een afweerreactie ontstaat tegen blaastumorcellen, met als uiteindelijk gevolg de afbraak van de tumorcellen.

Om meer zicht te krijgen op het verloop van het immunologische proces, is onderzoek gedaan naar urinemonsters van patienten die behandeld waren met BCG. Hiervan wordt verslag gedaan in **hoofdstuk 3**. Onderzocht zijn verschillende in de urine aanwezige immunologische componenten, zoals witte bloedcellen (*leucocyten*), geactiveerde witte bloedcellen (*lymfocyten*) en eiwitstoffen die worden geproduceerd door lymfocyten (*cytokines*).

Wat de witte bloedcellen aangaat: deze zijn bestudeerd met behulp van de *flow cytometrische analyse*, een methode waarmee cellen geselecteerd kunnen worden. De meest omvangrijke celpopulatie werd gevormd door *poly-*

morphenucleaire granulocyten (witte bloedcellen, die granula bevatten). Deze reactie wordt beschouwd als een a-specifieke reactie tegen bacterieen in het algemeen. Daarnaast waren verschillende soorten *mononucleaire leucocyten* in de urine aanwezig, zoals: *monocyten*, *macrofagen* en *T-lymfocyten*. Het aantal leucocyten in de urine bleek toe te nemen na toediening van BCG en dit kan worden uitgelegd als een uiting van cellulaire afweerreacties die optreden in de blaaswand.

Naast de granulocyten als grootste celpopulatie, zijn ook *T-lymfocyten* aangetoond na toediening van BCG. Omdat aangenomen wordt dat BCG vooral *T-lymfocyten* tot afweerreacties activeert, is dit celtype nader onderzocht. Gekeken is naar onderverdelingen binnen deze T-celpopulatie èn naar de staat van activiteit waarin deze lymfocyten verkeren na toediening van BCG. De meeste *T-lymfocyten* blijken van het CD4+ (helper/inducer) fenotype te zijn, de overige van het CD8+ (supressor/cytotoxische) fenotype. Verder zijn in de urine *natural killer cells* (NK-cellen) en *B-lymfocyten* aangetoond, zij het in zeer lage concentraties. Op de lymfocyten zijn IL-2 receptoren en HLA-DR antigenen waargenomen. Dit wijst er op dat de T-lymfocyten na toediening van BCG in de blaas geactiveerd zijn.

Ook is onderzocht of er tijdens de door BCG opgewekte ontstekingreactie *cytokines* (eiwitstoffen die door lymfocyten worden gemaakt) geproduceerd zijn. Daarbij zijn drie soorten *interleukines* aangetoond, namelijk: interleukine-1 (IL1), interleukine-2 (IL2), interleukine-6 (IL6), alsmede *tumornecrose factor-alpha* (TNFα). De aanwezigheid van IL2 wijst erop dat T-lymfocyten zijn geactiveerd. IL1 en IL6 kunnen, evenals TNFα, producten zijn van door BCG geactiveerde *monocyten/macrofagen*. Hoge concentraties van de interleukines en van TNFα zijn het meest frequent aangetroffen in urinemonsters die verzameld werden gedurende twee tot zes uur na de vierde, vijfde en zesde blaasspoeling met BCG.

De in hoofdstuk 3 beschreven onderzoeken leiden tot de volgende conclusies Instillatie van BCG geeft een a-specifieke ontstekingsreactie, waarbij mogelijk ook een specifieke T-cel reactie tegen mycobacterieen ontstaat. De combinatie van waargenomen leucocyten en cytokines speelt waarschijnlijk een belangrijke rol bij het antitumor effect van BCG bij blaastumoren. Mogelijk dat de aanwezigheid of afwezigheid van cytokines in de urine van met BCG behandelde patienten ook een voorspellende waarde heeft voor de effectiviteit van de behandeling.

Voorts blijkt de analyse van immunologische componenten in de urine van patienten die behandeld zijn met BCG een vruchtbare methode te zijn om meer inzicht te krijgen in de werking van deze therapie.

In **hoofdstuk 4** worden de resultaten gepresenteerd van een onderzoek dat tot doel had de effectiviteit van drie behandelingsmethoden van oppervlakkige blaastumoren te vergelijken. Het ging enerzijds om chemotherapie, waarbij *Mitomycine-C* via de blaas werd toegediend en anderzijds om intravesicale immunotherapie met twee verschillende BCG-stammen: de *Tice-stam* en de *RIVM-stam*. Behandeld werden 437 patienten met primaire en recidiverende oppervlakkige blaastumoren, inclusief patienten met *carcinoma in situ* in de blaas. Zij werden gevolgd gedurende een periode die varieerde tussen twee en 81 maanden, met een gemiddelde van 36 maanden. Na het verwijderen van alle zichtbare tumoren (door middel van *trans urethrale resectie*), kreeg een deel van de patienten Mitomycine-C toegediend. Eerst éénmaal per week gedurende vier achtereenvolgende weken en vervolgens een maandelijkse blaasspoeling gedurende zes maanden. De andere patienten werden behandeld met respectievelijk de *Tice-stam* en de *RIVM-stam*. Zij kregen, gedurende zes achtereenvolgende weken, eénmaal per week een dosering van 5×10^8 kolonievormende eenheden van één van beide stammen toegediend.

De vergelijking leverde de volgende resultaten op. Bij patienten met *papillaire tumoren* bleek behandeling met *Mitomycine-C* meer resultaat te hebben dan behandeling met *BCG-Tice* (het verschil is statistisch significant; p=0.01). De behandelingsresultaten van *Mitomycine-C* en *BCG-RIVM* vertoonden daarentegen geen significante verschillen. Verder konden er bij patienten met *carcinoma in situ* geen verschillen in effectiviteit tussen de drie methoden worden vastgesteld. Op grond van dit onderzoek kan worden geconcludeerd dat zes maanden intravesicale chemotherapie met *Mitomycine-C* effectiever is dan de intravesicale immunotherapie, waarbij gedurende zes weken de *BCG-Tice* wordt toegediend. Een bijkomende voordeel van *Mitomycine-C* is, dat het minder bijwerkingen heeft dan beide BCG-behandelingen.

Isoniazide wordt gebruikt bij de behandeling van patienten met actieve tuberculose. Op grond van theoretische inzichten valt te voorspellen dat preventieve toediening van Isoniazide een temperend effect kan hebben op de bijwerkingen van een BCG-behandeling. Maar omdat Isoniazide in feite bedoeld is om de BCG-bacterien te doden, kan het middel tevens van invloed zijn op de immunologische reactie na een BCG-behandeling. Dit laatste valt af te leiden uit experimenten met cavia's, die Isoniazide kregen toegediend na een intravesicale BCG-behandeling.

Het effect van preventieve toediening van Isoniazide op immunologische reacties, staat centraal in **hoofdstuk 5**. Een deel van de met BCG behandelde

patienten kreeg wèl en een ander deel géén Isoniazide toegediend. In welk opzicht verschilden beide groepen van elkaar? Uit het onderzoek bleek dat de concentratie van het 'vrije' (dat wil zeggen: niet aan een andere stof gebonden) Isoniazide in urinemonsters veel hoger was dan de minimale remmende concentratie die nodig is om tuberculosebacillen te doden (gemiddeld 38.0 ± 60.9 µgr Isoniazid/ml). Dit zou kunnen wijzen op tenminste een bacterie remmende potentie van de Isoniazide in de urine. Echter, laboratoriumstudies hebben aangetoond dat deze concentraties van Isoniazide in de urine de tuberculosebacillen niet effectief doden, zelfs niet in de concentratie van 150 µgr/ml gedurende 24 uur.

Na de vijfde en zesde BCG-spoeling is een duidelijke toename van de concentratie van de *cytokines* (IL2, IL6, IL8 en TNFα), IgG en IgA antilichamen tegen BCG en het aantal leucocyten in de urine waargenomen. De leucocyten bestonden voornamelijk uit granulocyten en verder uit monocyten en macrofagen en – in kleinere aantallen – T- en B-lymfocyten alsmede *natural killer cells*. Op de cytokines, de concentratie van IgA-antilichamen, en het aantal of de soorten leucocyten had Isoniazide geen effect.

Deze resultaten leiden tot de conclusie dat preventieve toediening van isoniazide bij mensen, anders dan bij cavia's, niet tot een verlaging van de afweerreactie leidt. Het is dan ook waarschijnlijk dat Isoniazide de antitumor effectiviteit van BCG bij de mens niet vermindert.

Als deze veronderstelling juist is en met behulp van Isoniazide de bijwerkingen van een BCG-therapie kunnen worden getemperd, dan zou preventieve toediening van Isoniazide een verbetering kunnen inhouden. Deze hypothese is klinisch getest en daarvan wordt verslag gedaan in **hoofdstuk 6.**

Tijdens een onderzoek in een aantal ziekenhuizen kreeg een deel van de patienten, elke keer als zij met *BCG-Tice* werden behandeld, ook 300 mg Isoniazide toegediend. Een ander deel van de patienten kreeg alleen *BCG-Tice*. Op het optreden van bijwerkingen bleek het toedienen van Isoniazide geen effect te hebben. De patienten die Isoniazide kregen hadden echter wel meer last van leverfunctiestoornissen, al waren deze van tijdelijke aard. De conclusie van het onderzoek luidt dan ook dat het preventieve gebruik van Isoniazide geen aanbeveling verdient.

Welke patient met een oppervlakkige blaastumor is nu gebaat bij de intravesicale BCG-therapie en welke niet? Dat is in de klinische praktijk niet eenvoudig uit te maken. De traditionele indicatoren (klinische en pathologische prognostische factoren zoals: multipliciteit van de tumor, recidief percentage van de tumor, tumorgradering en tumorstadium), waarvan urologen

bij deze afweging gebruik kunnen maken, lijken weinig betrouwbaar op het niveau van de individuele patiënt. Er bestaat dan ook behoefte aan nieuwe criteria.

In **hoofdstuk** 7 wordt de voorspellende waarde van conventionele indicatoren vergeleken met die van twee nieuwe, tumor-gerelateerde factoren: het *cel-adhesie molecule E-cadherine* en het *p53 eiwit*. De uitkomst van de vergelijking is dat conventionele indicatoren weliswaar van voorspellende waarde zijn maar zeker zouden moeten worden aangevuld met een nieuwe tumor-gerelateerde indicator. De aanwezigheid van een abnormale E-cadherine expressie in het tumorweefsel bleek namelijk van duidelijk voorspellende betekenis voor de tumorprogressie. Een abnormale expressie van het p53 eiwit bleek daarentegen in dit onderzoek geen voorspellende waarde te hebben.

Squamous and transitional elements in rat bladder carcinomas induced by N-butyl-N-4-hydroxy butyl-nitrosamine (BBN). A study of cytokeratine expression.
Chester J. Herman, **Peter D.J. Vegt**, Frans M.J. Debruyne, G. Peter Vooys, Frans C.S. Ramaekers.
AJP. 419-426, 1985.

Presence of interleukine-2 in urine of superficial bladder cancer patients after intravesical treatment with bacillus Calmette-Guérin.
W.H. de Jong, E.C. de Boer, A.P.M. van der Meijden, **P.D.J. Vegt**, P.A. Steerenberg, F.M.J. Debruyne and E.J. Ruitenberg.
Can. Imm. Immunoth. 31: 182-186, 1990.

Leucocytes in the urine after intravesical BCG-treatment for superficial bladder cancer.
E.C. De Boer, W.H. De Jong, A.P.M. van der Meijden, P.A. Steerenberg, F. Witjes, **P.D.J. Vegt**, F.M.J. Debruyne and E.J. Ruitenberg.
Urol.Res. 19: 45-50, 1991.

Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guérin.
E.C. De Boer, W.H. De Jong, A.P.M. Van Der Meijden, P.A. Steerenberg, J.A. Witjes, **P.D.J. Vegt**, F.M.J. Debruyne and E.J. Ruitenberg.
Cancer Immunol. Immunother. 33: 411-416, 1991.

Induction of urinary IL1, IL2,IL6 and TNF during intravesical immunotherapy with BCG in superficial bladder cancer.
E.C. De Boer, W.H. De Jong, P.A. Steerenberg, L.A. Aarden, E. Tetteroo, E.R. De Groot, A.P.M. Van der Meijden, **P.D.J. Vegt**, F.M.J. Debruyne and E.R. Ruitenberg.
Cancer Immunol. Immunother. 34: 306-312, 1992.

Effects of Isoniazid (INH) on the BCG-induced local immune response after intravesical BCG-therapy for superficial bladder cancer.
M.J.J.G. Stassar, **P.D.J. Vegt**, P.A. Steerenberg, A.P.M. van der Meijden, H.D. Meiring, M.Dessens-Kroon, H.G.M. Geertzen and W. den Otter.
Urological Research 22: 177-184, 1994.

Effects of sequential intravesical administration of mitomycin C and bacillus Calmette-Guérin on the immune response in the guinea pig bladder.
L.T.M. Balemans, **P.D.J. Vegt**, P.A. Steerenberg, E.C. de Boer, A. van Swaaij, R.E.M. de Vries, A.P.M. van der Meijden, W. den Otter.
Urological Research 22: 239-245, 1994.

BCG in superficial bladder cancer. Consensus and controversies.
P.D.J. Vegt, F.M.J. Debruyne, A.P.M. van der Meijden.
Eur.Urol. 27: 89-95, 1995.

A randomized study of intravesical mitomycin-c , BCG-Tice and BCG-RIVM treatment in pTa-pT1 papillary carcinoma and carcinoma in situ of the urinary bladder.
P.D.J. Vegt, J.A. Witjes, W.P.J. Witjes, W.H. Doesburg, F.M.J. Debruyne, A.P.M. van der Meijden.
J.Urol. 153: 929-933,1995.

Does Isoniazid reduce side effects of intravesical Bacillus Calmette-Guérin therapy in superficial bladder cancer? Results of EORTC-protocol 30911.
Peter D.J. Vegt, Adrian P.M. van der Meiden, Richard Sylvester, Maurizio Brausi, Wolfgang Holtl, Christine de Balincourt and other members of the EORTC GU-group.
J.Urol. 157: 1246-1249, 1997.

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Peter Dave Johan Vegt (De Bilt, 1950) ging in 1969 geneeskunde studeren aan de Rijksuniversiteit Groningen. Als student-assistent werkte hij op de afdeling urologie van het academisch ziekenhuis te Groningen. De afdeling werd in die tijd geleid door Prof. P.W. Boer. In 1995 legde hij het artsexamen af.

Van 1976 tot 1978 werkte hij op Curaçao in het St. Elizabeth ziekenhuis op de afdeling interne geneeskunde, destijds geleid door prof. dr. E.A.C. Saleh. Terug in Nederland werd hij aangesteld als arts-assistent in opleiding bij de afdeling algemene heelkunde van het St. Hippolytus ziekenhuis te Delft, met als opleider destijds dr. W.M. Oosterwijk. Prof. dr. F.M.J. Debruyne, verbonden aan de Katholieke Universiteit Nijmegen, nam hem daarna in 1981 aan als uroloog in opleiding. In 1983 vervolgde hij de opleiding bij prof. dr. R. A. Janknegt van het Grootziekenhuis in Den Bosch.

P.D.J. Vegt werd in 1984 geregistreerd als uroloog en kon in datzelfde jaar aan de slag in het academisch ziekenhuis van de Katholieke Universiteit Nijmegen. Vanaf 1985 is hij als uroloog verbonden aan het Rijnlandziekenhuis te Leiderdorp.

Tenslotte is hij de trotse vader van drie geweldige dochters: Liesbeth, Fabienne en Willemijn.

STELLINGEN

behorend bij het proefschrift

IMPROVEMENTS OF BCG-IMMUNOTHERAPY
IN SUPERFICIAL BLADDER CANCER

van Peter D.J. Vegt

Nijmegen, 16 september 1997

1. Intravesicale immunotherapie met BCG is effectief ten aanzien van het voorkomen van recidief blaastumoren alsmede de behandeling van oppervlakkige blaastumoren.
   Dit proefschrift.

2. Analyse van immunologische producten in de urine van patiënten na intravesicale BCG-toediening kunnen de inzichten in het werkingsmechanisme van de antitumoractiviteit van BCG verruimen.
   Dit proefschrift.

3. Intravesicale chemotherapie is bij bepaalde oppervlakkige blaastumoren even effectief als intravesicale immunotherapie.
   Dit proefschrift.

4. Het nut van het adjuvant toedienen van Isoniazid ter vermindering van de bijwerkingen van intravesicale immunotherapie is niet aangetoond.
   Dit proefschrift.

5. Profylactisch toedienen van Isoniazid tijdens de behandeling met BCG geeft passagière leverfunctiestoornissen.
   Dit proefschrift.

6. Kwantitatieve cytologie (Quanticyt) is een waardevol onderzoek bij de followup van patiënten met oppervlakkige blaastumoren.

7. Een abnormale E-cadherin expressie in blaastumorweefsel is een prognostische tumormarker met een hoge specificiteit ten aanzien van de kans op tumorprogressie.
   Dit proefschrift.

8. Met de toegenomen mogelijkheden voor de medicamenteuze behandeling van obstructieve prostaatklachten zijn de resultaten van de operatieve therapieën bij patiënten met deze klachten verbeterd.

9. Het roken van sigaretten bevordert het ontstaan van blaaskanker.

10. Omdat een voorste kruisband in het kniegewricht een natuurlijke intrinsieke torsie heeft, zal voor een zo optimaal mogelijk resultaat van een voorste kruisbandreconstructie de te plaatsen nieuwe kruisband eveneens getordeerd moeten worden.
    Prof. dr. Th.J.G. van Rens.

11. Voor het adequaat kunnen functioneren van een Vereniging Medische Staf in een ziekenhuis is een kernstaf met gemandateerde leden een vereiste.

12. Een coöperatieve vereniging van vrijgevestigde medisch specialisten is een noodzakelijk kwaad.

13. Promoveren kun je leren.
    Dr. P.A. Vegt.